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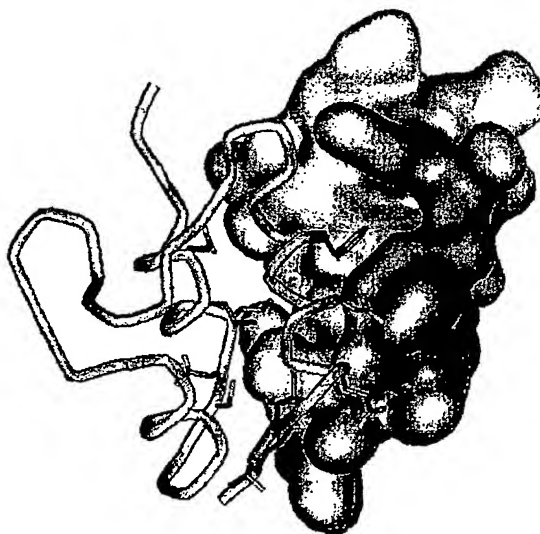
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(54) Title: **MUTANTS OF IGF BINDING PROTEINS AND METHODS OF PRODUCTION OF ANTAGONISTS THEREOF**



(57) Abstract: The present invention provides a crystal suitable for X-ray diffraction, comprising a complex of insulin-like growth factor I or II (IGF) and a polypeptide consisting of the amino acids 39-91 of IGFBP-1, the amino acids 55-107 of IGFBP-2, the amino acids 47-99 of IGFBP-3, the amino acids 39-91 of IGFBP-4, the amino acids 40-92 of IGFBP-5, or the amino acids 40-92 of IGFBP-6 or a fragment thereof consisting at least of the 9th to 12th cysteine of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, or IGFBP-5 or at least of the 7th to 10th cysteine of IGFBP-6; methods for the determination of the atomic coordinates of such a crystal; IGFBP mutants with enhanced binding affinity for IGF-I and/or IGF-II, and methods to identify and optimize small molecules which displace IGFs from their binding proteins.

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Mutants of IGF binding proteins and methods of production of antagonists thereof

The present invention relates to a complex of an IGF binding protein fragment (IGFBP) with IGF, its uses and to novel IGFBP mutants with a higher affinity than natural IGFBPs for IGF as well as to methods for the production of antagonists for IGFBPs which hinder or reverse complex formation between IGFBPs and IGF.

5 Introduction

Insulin-like growth factors I and II (hereafter also referred to as IGFs or IGF) are members of the insulin superfamily of hormones, growth factors and neuropeptides whose biological actions are achieved through binding to cell surface receptors. IGF actions are regulated by IGF binding proteins (IGFBPs) that act as transporters of IGFs, protect them from degradation, limit their binding to receptors, and maintain a "reservoir" of biologically inactive IGF (Martin, J.L., and Baxter, R.C., IGF binding proteins as modulators of IGF actions; in: Rosenfeld, R.G., and Roberts, C.T. (eds.), The IGF system, Molecular Biology, Physiology, and Clinical Applications (1999), Humana Press, Totowa, pp. 227-255; Jones, J.L., and Clemmons, D.R., Endocr. Rev. 12 (1995) 10-21; Khandwala, H.M., et al., Endocr. Rev. 21 (2000) 215-244; Hwa, V., et al., The IGF binding protein superfamily, In: Rosenfeld, R.G., and Roberts, C.T. (eds.), The IGF system, Molecular Biology, Physiology, and Clinical Applications (1999), Humana Press, Totowa, pp. 315-327). The IGF and growth hormone (GH) axis plays a large part in regulating fetal and childhood somatic growth and several decades of basic and clinical research have demonstrated that it also is critical in maintaining neoplastic growth (Khandwala, H.M., et al., Endocr. Rev. 21 (2000) 215-244). High circulating IGF-I concentrations may also be an important determinant of cancer incidence (Hankinson, S.E., et al., Lancet 351 (1998) 1393-1396; Holly, J., Lancet 351 (1998) 1373-1374; Wolk, A., Lancet 356 (2000) 1902-1903). Virtually every level of the IGF system mediating response on the tumor tissues (IGFs, IGFBPs, IGF receptors) can be targeted for therapeutic approaches (Khandwala, H.M., et al., Endocr. Rev. 21 (2000) 215-244; Fanayan, S., et al., J. Biol. Chem. 275 (2000) 39146-39151; Imai, Y., et al., J. Biol. Chem. 275 (2000) 18188-18194). It should also be mentioned here that IGFBP-3 has IGF-independent anti-proliferative and proapoptotic effects (Wetterau, L.A., et al., Mol.

Gen. Metab. 68 (1999) 161-181; Butt, A.J., et al., J. Biol. Chem. 275 (2000) 39174-39181).

IGF-I and IGF-II are 67% identical single polypeptide chains of 70 and 67 amino acids, respectively, sharing with insulin about 40% sequence identity and presumed structural homology. The first 29 residues of IGFs are homologous to the B-chain of insulin (B region, 1-29), followed by 12 residues that are analogous to the C-peptide of proinsulin (C region, 30-41), and a 21-residue region that is homologous to the A-chain of insulin (A region, 42-62). The carboxy-terminal octapeptide (D region, 63-70) has no counterpart in insulins and proinsulins (Murray-Rust, J., et al., BioEssays 14 (1992) 325-331; Baxter, R.C., et al., J. Biol. Chem. 267 (1992) 60-65). The IGFs are the only members of the insulin superfamily in which the C region is not removed proteolytically after translation. The 3D structure of insulin has been studied intensively since the first crystal structure determination in the 1960s (Adams, M.J., et al., Nature 224 (1969) 491-492). There are now structures of insulins in several oligomeric states, for insulins crystallized in different solvent conditions, and for many variants from different species and chemical modifications. This is in stark contrast to IGFs (and proinsulins) for which no high definition structure has been available prior to this report. Instead, the tertiary structure of IGF-I has been modeled after porcine insulin (Blundell, T.L., Proc. Natl. Acad. Sci. USA 75 (1978) 180-184). 2D NMR studies of IGF-I have confirmed that the solution structure is consistent with the model (Cooke, R.M., et al., Biochemistry 30 (1991) 5484-5491; Sato, A., et al., Int. J. Pept. Protein Res. 41 (1993) 433-440). However, NMR studies of IGF-I have yielded structures only of low resolution, probably because IGF-I is soluble at the concentrations required for NMR only at pH values below 3 (Cooke, R.M., et al., Biochemistry 30 (1991) 5484-5491; Sato, A., et al., Int. J. Pept. Protein Res. 41 (1993) 433-440). More recently, better defined structures have been obtained for IGF-II (Terasawa, H., et al., EMBO J. 13 (1994) 5590-5597; Torres, A.M., et al., J. Mol. Biol. 248 (1995) 385-401) and for a Glu-3 to Arg variant of IGF-I (long-[Arg3]IGF-I) that additionally possesses a 13-amino acid extension at the N-terminus (Laajoki, L.G., et al., J. Biol. Chem. 275 (2000) 10009-10015).

IGFBPs (insulin-like growth factor binding proteins -1 to -6) are proteins of 216 to 289 residues, with mature IGFBP-5 consisting of 252 residues (Wetterau, L.A., et al., Mol. Gen. Metab. 68 (1999) 161-181). All IGFBPs share a common domain

organization. The highest conservation is found in the N- (residues 1 to ca. 100) and C- (from residue 170) terminal cysteine rich regions. Twelve conserved cysteines are found in the N-terminal domain and six in the C-terminal domain. The central, weakly conserved part (L-domain) contains most of the cleavage sites for specific proteases (Chernausek, S.D., et al., J. Biol. Chem. 270 (1995) 11377-11382). Several different fragments of IGFBPs have been described and biochemically characterized so far (Mazerbourg, S., et al., Endocrinology 140 (1999) 4175-4184). Mutagenesis studies suggest that the high affinity IGF binding site is located in the N-terminal domain (Wetterau, L.A., et al., Mol. Gen. Metab. 68 (1999) 161-181; Chernausek, S.D., et al., J. Biol. Chem. 270 (1995) 11377-11382) and that at least IGFBP-3 and IGFBP-2 contain two binding determinants, one in the N- and one at the C-terminal domains (Wetterau, L.A., et al., Mol. Gen. Metab. 68 (1999) 161-181). Recently, a group of IGFBP-related proteins (IGFBP-rPs) which bind IGFs with lower affinity than IGFBPs have been described (Hwa, V., et al., The IGF binding protein superfamily, In: Rosenfeld, R.G., and Roberts, C.T. (eds.), The IGF system, Molecular Biology, Physiology, and Clinical Applications (1999), Humana Press, Totowa, pp. 315-327). IGFBPs and IGFBP-rPs share the highly conserved and cysteine-rich N-terminal domain which appears to be crucial for several biological actions, including their binding to IGFs and high affinity binding to insulin (Hwa et al., 1999). N-terminal fragments of IGFBP-3, generated for example by plasma digestion, also bind insulin and physiologically are thus likely relevant for insulin action. Beyond the N-terminal domain, there is a lack of sequence similarity between the IGFBPs and IGFBP-rPs.

The sequences of human IGFBP-1 to -6 are described in detail in the SwissProt Database (<http://www.expasy.ch>) and identified by the following Accession Nos.:

Name	Accession No.
IGFBP-1	P 08833
IGFBP-2	P 18065
IGFBP-3	P 17936
IGFBP-4	P 22692
IGFBP-5	P 24593
IGFBP-6	P 24592

The amino acid positions described in the following refer to the sequence of the mature forms the human IGF binding proteins (sequence after removal of the signaling peptide starts with amino acid in position 1, see also Tables 1 to 6).

5 The association of insulin-like growth factors with neoplasia indicates that inhibition of the IGF signaling pathway in tumors might be a successful strategy in cancer therapy. Such modulation might be accomplished, for example, through exogenous administration of recombinant inhibitory IGFBPs and effective fragments thereof. Additionally, tumor cell IGFBP production, inhibition or degradation may be controlled by agents such as tamoxifen and ICI 182,780 that
10 modify tumor IGFBP production (Khandwala, H.M., et al., *Endocr. Rev.* 21 (2000) 215-244). The consequent alteration in IGFBP-3 levels appears in certain instances to inhibit IGF-I-stimulated cell proliferation (Khwandala et al., 2000). There is also recent evidence that IGFBP-3 may be a p53-independent effector of apoptosis in breast cancer cells via its modulation of the Bax:Bcl-2 protein ratio (Butt, A.J., et al.,
15 *J. Biol. Chem.* 275 (2000) 39174-39181; Wetterau, L.A., et al., *Mol. Gen. Metab.* 68 (1999) 161-181).

IGFBPs show a significant inhibition of tumor cell proliferation in vitro but only very high doses result in inhibition of tumor growth in vivo (van den Berg, C.L., et al., *Eur. J. Cancer* 33 (1997) 1108-1113). Van den Berg therefore covalently coupled
20 IGFBP-1 to polyethylene glycol, which leads to a prolonged serum half-life. However, the inhibitory effects of the pegylated IGFBP-1 is still not sufficient for therapeutic intervention in humans because only partial response is observed even if pegylated IGFBP-1 is given in doses of 1 mg/dose daily in mice. This corresponds to a dose of 50 mg/kg x day which can not be administered to humans
25 by established procedures and can not be produced economically.

IGF releasing peptides are described by Loddick, S.A., et al., *Proc. Natl. Acad. Sci. USA* 95 (1998) 1894-1898 and Lowman, H.B., et al., *Biochemistry* 37 (1998) 8870-8878. The described molecules which are able to displace IGFs from their binding proteins are either mutants of IGF-I which bind to IGFBPs but are not able to
30 stimulate the IGF-IR or a 14 amino acid peptide with similar properties derived from a phage-display library. The biological activities of the peptides were shown by administration either by injection into the lateral ventricle of the brain or infused by a minipump.

5 Mutagenesis studies for IGFs indicated that IGF amino acid residues Glu 3, Thr 4, Gln 15 and Phe 16 of IGF-I and Glu 6, Phe 48, Arg 49 and Ser 50 in IGF-II are important for binding to IGFBPs (Baxter, R.C., et al., J. Biol. Chem. 267 (1992) 60-65; Bach, L.A., et al., J. Biol. Chem. 268 (1993) 9246-9254; Luethi, C., et al., Eur. J. Biochem. 205 (1992) 483-490; Jansson, M., et al., Biochemistry 36 (1997) 4108-4117). Baxter et al. (1992) suggested that the IGF-I amino acid residues Glu 3, Thr 4, Gln 15 and Phe 16 are crucial for interaction with IGFBP-3, whereas residues Phe 49, Arg 50 and Ser 51 are of secondary importance. It also was suggested that Phe 26 of IGF-II plays a role in changing the local structures of IGFs but does not
10 directly bind to IGFBPs (Terasawa, H., et al., EMBO J. 13 (1994) 5590-5597).

Kalus, W., et al., in EMBO J. 17 (1998) 6558-6572, describe proteolytic studies of human IGFBP-5 and the cloning and expressing of the domain of IGFBP-5 between residues 40-92 (mini-IGFBP-5); this domain binds IGF-I and IGF-II with K_D values of 37 nM and 6 nM, respectively, as well as the determination of the solution
15 structure of uncomplexed mini-IGFBP-5 by NMR. Kalus et al. identified some IGF binding sites which are residues Val49, Tyr50, Pro62 and Lys68 to Leu75 of IGFBP-5.

Imai, Y., et al., in J. Biol. Chem. 275 (2000) 18188-18194, describe an IGFBP-3 variant and an IGFBP-5 variant, each with a five-fold substitution pattern at amino acid positions hypothesized by Kalus et al. as IGF binding sites. Imai et al. found
20 that a substantial alteration of the amino acid residues simultaneously at positions 68, 69, 70, 73 and 74 results in a 1000-fold or larger reduction in the affinity for IGF-I in relation to the affinity of wild-type IGFBP-5.

Conover, C.A., et al., in J. Biol. Chem. 270 (1995) 4395-4400, describe protease-resistant mutants of IGFBP-4. All four IGFBP-4 mutants around the putative cleavage site at Met135-Lys136 and the wild-type protein bind IGFs with equivalent
25 affinities.

Byun, D., et al., in J. Endocrinology 169 (2001) 135-143, postulate several regions involved in IGF binding by IGFBP-4. Deletion of segments Leu72-Ser 91 or Leu72-His74 results in loss of IGF binding. Also mutation of certain cysteine residues
30 significantly reduces the binding of IGFs.

Thus, these described mutant forms of insulin-like growth factor binding proteins have reduced or equivalent affinities for IGF-I and/or IGF-II. Mutants of IGFBPs with a significantly higher affinity and a therefore improved effectiveness have not been known heretofore and there exists a need for such molecules as well as for methods for identifying IGFBP antagonists.

Summary of the Invention

The invention provides a crystal suitable for X-ray diffraction, comprising a complex of insulin-like growth factor I or II and a polypeptide consisting of the amino acids 39-91 of IGFBP-1, the amino acids 55-107 of IGFBP-2, the amino acids 47-99 of IGFBP-3, the amino acids 39-91 of IGFBP-4, the amino acids 40-92 of IGFBP-5, or the amino acids 40-92 of IGFBP-6 or a fragment thereof consisting at least of the 9th to 12th cysteine of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, or IGFBP-5 or at least of the 7th to 10th cysteine of IGFBP-6 (such polypeptides and fragments are hereinafter also referred to as "mini-IGFBPs").

Such a crystal is suitable for determining the atomic coordinates of the binding sites of IGF-I, IGF-II, and IGFBPs, and therefore allows the optimization of these molecules to identify and improve stabilizing interactions between IGF-I or IGF-II and IGFBPs. Preferably, the crystal effectively diffracts X-ray for the determination of the atomic coordinates of said complex to a resolution of 1.5 to 3.5 Å. The crystal is arranged in the cubic space group P2₁3 having unit cell dimensions of 74.385 Å x 74.385 Å x 74.385 Å.

The invention further provides a method for producing a crystal suitable for X-ray diffraction, comprising

- (a) contacting IGF-I or IGF-II with a polypeptide consisting of the amino acids 39-91 of IGFBP-1, the amino acids 55-107 of IGFBP-2, the amino acids 47-99 of IGFBP-3, the amino acids 39-91 of IGFBP-4, the amino acids 40-92 of IGFBP-5, or the amino acids 40-92 of IGFBP-6 or a fragment thereof consisting at least of the 9th to 12th cysteine of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, or IGFBP-5 or at least of the 7th to 10th cysteine of IGFBP-6, to form a complex which exhibits restricted conformation mobility, and
- (b) obtaining a crystal from the complex so formed suitable for X-ray diffraction.

Using this crystal, the atomic coordinates of the complex can be determined.

The invention further comprises a method for identifying a mutant of IGFBP or a mutant of a fragment thereof consisting at least of the 9th to 12th cysteine of
5 IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, or IGFBP-5 or at least of the 7th to 10th cysteine of IGFBP-6, and having enhanced binding affinity for IGF-I and/or IGF-II comprising

- 10 (a) constructing a three-dimensional structure of the complex of IGF-I or IGF-II and a polypeptide consisting of the amino acids 39-91 of IGFBP-1, the amino acids 55-107 of IGFBP-2, the amino acids 47-99 of IGFBP-3, the amino acids 39-91 of IGFBP-4, the amino acids 40-92 of IGFBP-5, or the amino acids 40-92 of IGFBP-6 or a fragment thereof consisting at least of the 9th to 12th cysteine of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, or IGFBP-5 or at least of
15 the 7th to 10th cysteine of IGFBP-6, based on the atomic coordinates of a crystal consisting of IGF-I or IGF-II and said polypeptide;
- (b) employing said three-dimensional structure and modeling methods to identify said mutant in which an amino acid residue within a distance of 5 Å to a hydrophobic amino acid residue of IGF-I or IGF-II is modified in that
20 the hydrophobic interaction between IGF-I or IGF-II and said mutant of IGFBP is enhanced;
- (c) producing said mutant;
- (d) assaying said mutant to determine said enhanced binding affinity for IGF.

25 The invention further comprises a method for identifying a mutant of IGFBP-5 with enhanced binding affinity for IGF-I, said method comprising

- (a) constructing a three-dimensional structure of the complex of IGF-1 and IGFBP-5 defined by the atomic coordinates shown in Figs. 5 and 6;
- 30 (b) employing said three-dimensional structure and modeling methods to identify an amino acid residue in IGFBP-5 within a distance of 5 Å or shorter to an amino acid residue of IGF-I, wherein said residue of IGFBP-5 can be modified hydrophobically in that the hydrophobic interaction between IGF and IGFBP-5 is enhanced;
- 35 (c) producing said mutant;

(d) assaying said mutant to determine said enhanced binding affinity for IGF.

5 The amino acid residue(s) in which IGFBP(s) is/are modified is/are preferably selected from the amino acids 39-91 of IGFBP-1, the amino acids 55-107 of IGFBP-2, the amino acids 47-99 of IGFBP-3, the amino acids 39-91 of IGFBP-4, the amino acids 49-92 of IGFBP-5, or the amino acids 40-92 of IGFBP-6.

Especially preferred IGFBP mutants are modified at amino acid residues 49, 70 and/or 73 corresponding to IGFBP-5 sequence alignment and according to Table 7.

10 The invention therefore provides mutant IGFBPs ("IGFBPs" as used herein means IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5 and/or IGFBP-6) with enhanced affinity (preferably about 3-fold to 10-fold increased affinity to the corresponding wild-type IGFBP) for IGF ("IGF" as used herein means IGF-I and/or IGF-II), improved inhibitory potency for the activity of IGF in vitro and in vivo and therefore improved therapeutic effectiveness.

15 The invention further provides a method for identifying a compound capable of binding to IGFBP, comprising

- 20 (a) constructing a three-dimensional structure of a complex of IGF-I or IGF-II and a polypeptide consisting of the amino acids 39-91 of IGFBP-1, amino acids 55-107 of IGFBP-2, amino acids 47-99 of IGFBP-3, amino acids 39-91 of IGFBP-4, amino acids 40-92 of IGFBP-5, amino acids 40-92 of IGFBP-6 or a fragment thereof consisting at least of the 9th to 12th cysteine of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, or IGFBP-5 or at least of the 7th to 10th cysteine of IGFBP-6, based on the atomic coordinates of a crystal consisting of IGF - I and said IGFBP;
- 25 (b) employing said three-dimensional structure and modeling methods to identify a compound forming a complex with said IGFBP by hydrophobic binding with amino acids 49, 50, 70, 71 and 74 in the case of IGFBP-5 and in the case of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4 and IGFBP-6 with
- 30 corresponding amino acids according to Table 7;
- (c) producing said compound;
- (d) determining the binding between the compound and IGFBP.

The invention further provides a method of inhibiting the binding of IGF to the IGFBP in a subject, preferably a human subject, comprising administering an effective amount of an above-described mutant of IGFBP to the subject.

Detailed Description of the Invention

5 The present invention provides methods for co-crystallizing IGF-I or IGF-II with a truncated N-terminal fragment of IGFBP, preferably of IGFBP-5 (mini-IGF), where the crystals diffract X-rays with sufficiently high resolution to allow determination of the three-dimensional structure of said complex, including atomic coordinates. The three-dimensional structure (e.g. as provided on computer-readable media) is
10 useful for rational drug design of IGFBP mutants with modified affinity for IGF-I or IGF-II, preferably with an improved affinity. There is specifically provided a method for co-crystallizing IGF-I with a polypeptide consisting of an isolated folded domain of IGFBPs (mini-IGFBPs), which is formed by the amino acids between the 9th and the 12th cysteine of IGFBP-1 to IGFBP-5 or the 7th and 10th
15 cysteine of IGFBP-6 and additionally including up to 7 amino acids N-terminal of this fragment and up to 5-20 amino acids C-terminal to this fragment. The amino acids 39-91 of BP-1, the amino acids 55-107 of IGFBP-2, the amino acids 47-99 of IGFBP-3, the amino acids 39-91 of IGFBP-4, the amino acids 40-92 of IGFBP-5, or the amino acids 40-92 of IGFBP-6 or fragments thereof are especially suitable to
20 form a complex with IGF-I or IGF-II which exhibits restricted conformational mobility and high suitability for X-ray diffraction.

Such a complex co-crystallizes in a manner sufficient for the determination of atomic coordinates by X-ray diffraction. The crystal effectively diffracts X-ray for the determination of the atomic coordinates of the complex to a resolution of 1.5 or
25 at least better (less) than 3.5 Å. Said IGFBP fragments are able to form a compact and globular structure whose scaffold is secured by an inside packing of two cysteine bridges and stabilized further by a three-stranded β-sheet. The folded fragments are still able to bind IGF-I and IGF-II with high affinities. Other forms of the IGFBPs such as full-length IGFBPs, the isolated C-terminal domain of IGFBPs
30 or fragments without N-terminal truncation do not co-crystallize with IGF in a suitable manner for X-ray-based determination of the structure at high resolution.

Knowledge of the crystal structure enables the production of specific IGFBP mutants which develop improved interaction with, thereby exhibiting enhanced affinity for, IGF and, as a consequence, have improved therapeutic efficacy as IGF antagonists. Such IGFBP mutants with increased affinity for IGF are capable of preventing the formation of the complex between naturally occurring IGF and IGF-I receptor (IGF-IR) in vitro and in vivo and, thereby, of effecting a decrease in the concentration of biologically active, free IGF. Such rational designed IGF antagonists are therefore capable of inhibiting tumor growth and inducing apoptosis in tumor cells more efficient than natural IGFBPs. As a result, lower doses of the optimal designed IGFBP mutants with enhanced affinity are needed for achieving an effect comparable to that of naturally occurring IGFBPs.

A further embodiment of the invention is the identification and optimization of non-proteinaceous compounds which bind to the IGF binding site of IGFBPs and prevent the formation of an inhibitory complex between IGFs and IGFBPs and therefore activates the anabolic action of IGF. Such "IGF-releasing compounds" can be identified according to the invention on the basis of the crystal data, using protein-ligand docking programs such as FlexX (Kramer, B., et al., *Proteins: Structure, Functions and Genetics* 37 (1999) 228-241).

The X-ray diffraction patterns of the invention have a sufficiently high resolution to be useful for three-dimensional modeling of an IGF releasing compound. Preferably, the resolution is in the range of 1.5 to 3.5 Å, preferably 1.5 to 3.0 Å. Three-dimensional modeling is performed using the diffraction coordinates from these X-ray diffraction patterns. The coordinates are entered into one or more computer programs for molecular modeling as known in the art. Such molecular modeling can utilize known X-ray diffraction molecular modeling algorithms or molecular modeling software to generate atomic coordinates corresponding to the three-dimensional structure of at least one IGF releasing compound.

Such a compound shows affinity for IGFBP based on stereochemical complementary relative to naturally occurring IGFs. Such stereochemical complementary according to the present invention is characterized by a molecule that matches intra-site surface residues that form the contours of IGFBPs as enumerated by the coordinates set out in Figs. 5 and 6. The residues that define the contours are depicted in Figs. 5 and 6. Matching according to the invention means

that the identified atoms or atom groups interact with the IGFBP surface residues, for example via hydrogen bonding or by enthalpy-reduced van der Waals interactions which prevent or reduce the interaction between IGFBP and IGFs and thereby promote the release of the biologically active compound from the binding site. In general, the design of a molecule possessing stereochemical complementary to the contours of IGFBPs can be accomplished by means of techniques that optimize either chemically or geometrically the fit between a molecule and a target receptor. Known techniques of this sort are reviewed by Sheridan, R.P., and Venkataraghavan, R., *Acc. Chem. Res.* 20 (1987) 322; Goodford, P.J., *J. Med. Chem.* 27 (1984) 557; Verlinde, C., and Hol, W., *Structure* 2 (1994) 577; and Blundell, T.L. et al., *Nature* 326 (1987) 347. The design of optimized IGFBP ligands based on the invention is preferably done by the use of software such as GRID (Goodford, P.J., *J. Med. Chem.* 28 (1985) 849-857), a program that determines probable interaction sites between probes with various functional group characteristics and the protein surface - is used to analyze the surface sites to determine structures of similar inhibiting proteins or compounds.

The program DOCK (Kuntz, I.D., et al., *J. Mol. Biol.* 161 (1982) 269-288) can also be used to analyze an active site or ligand binding site and suggest ligands with complementary steric properties. Several methodologies for searching three-dimensional databases to test pharmacophore hypotheses and select compounds for screening are available. These include the program CAVEAT (Bacon et al., *J. Mol. Biol.* 225 (1992) 849-858) which uses databases of cyclic compounds which can act as spacers to connect any number of chemical fragments already positioned in the active site. The program LUDI (Bohm, H.J., et al., *J. Comput. Aided Mol. Des.* 6 (1992) 61-78 and 593-606) defines interaction sites into which both hydrogen bonding and hydrophobic fragments fit.

Programs suitable for searching three-dimensional databases to identify also non-proteinaceous molecules bearing a desired pharmacophore include: MACCS-3D and ISIS/3D (Molecular Design Ltd., San Leandro, CA), ChemDBS-3D (Chemical Design Ltd., Oxford, U.K.), and Sybyl/3DB Unity (Tripos Associates, St. Louis, MO).

Programs suitable for pharmacophore selection and design include: DISCO (Abbott Laboratories, Abbott Park, IL), Catalyst (Bio-CAD Corp., Mountain View, CA), and ChemDBS-3D (Chemical Design Ltd., Oxford, U.K.).

5 Databases of chemical structures are available from a number of sources including Cambridge Crystallographic Data Centre (Cambridge, U.K.) and Chemical Abstracts Service (Columbus, OH).

De novo design programs include Ludi (Biosyrn Technologies Inc., San Diego, CA), Sybyl (Tripos Associates) and Aladdin (Daylight Chemical Information Systems, Irvine, CA).

10 Those skilled in the art will recognize that the design of such compounds may require slight structural alteration or adjustment of a chemical structure designed or identified using the methods of the invention.

15 Non-proteinaceous compounds and IGFBP mutants with increased binding affinity for IGF can be identified by incubating said compounds or mutants with an IGF-I/IGFBP-5 complex and measuring the binding of released IGF-I to IGF-I receptor expressing cells. Due to the binding of IGF-I to its cell-bound receptor, the receptor is activated and autophosphorylated. Alternatively, radiolabeled IGF-I can be used and its binding to its receptor after release from the complex can be determined.

20 Formation of the IGF-I mini-IGFBP-5 complex buries a binding surface totalling about 550 Å². Of the eleven IGFBP-5 amino acid residues within 5 Å of IGF, six are hydrophobic, three of which are surface-exposed leucines and valines and are of primary importance for hydrophobic interaction to IGFs (Figures 1 to 4). On the IGF side, four of the eleven amino acid residues within 5 Å of mini-IBFBP-5 are
25 hydrophobic (Figures 1 to 4).

The IGFBPs bind to IGF-I and IGF-II by presenting a binding surface complementary to that of IGF. The IGF binding surface consists of a relatively flat hydrophobic surface, a small hydrophobic depression, two hydrophobic protruberances, and surrounding polar residues. Identification of the IGF binding
30 surface itself (Figure 3) enables the design of binding partners in general, and

optimization of known binding partners in particular. General binding partners will have at least two of the following features 1 to 4:

1. Non-polar atoms lying approximately in a plane defined by atoms Leu74 CD1 and CD2, Val49 CG1 and CG2, Leu70 CB, and Tyr 50 CB, within a perimeter defined by IGF residues Glu9, Glu3, Leu54, Phe 16 and by BP5 atom Tyr 50 OH and depicted in Figure 3 such that they present an approximately planar and hydrophobic molecular surface of at least 20 square Angstroms.
2. A non-polar atom or atoms near the positions of Leu 70 CG, CD1 relative to IGF, filling the depression of IGF as seen in the complex structure.
3. Hydrophobic and/or aromatic interactions with the side chains of Phe16, Val17, and/or Leu54 of IGF as defined by a net buried surface area in the complex of at least 20 square Angstroms.
4. Polar (hydrogen bonding and/or charge complementary) interactions, either directly or via bridging solvent molecules, with one or more of the following IGF atoms: Asp12 OD1,2; Glu9 OE1,2; Glu3 OE1,2; Glu58 OE1,2; Thr4 O,OG1; Cys52O; Ser51 OG; Asp53OD1,2; Arg55NH1,2,NE; Arg21NH1,2,NE; Val17O; Cys18O; Asp20OD1,2,N; Gln15O,OD1,ND2.

Abbreviations: Letters corresponding to standard amino acid atom naming (according to the International Union of Physicists and Chemists—IUPAC—naming).

CG:	Carbon C γ
CB:	Carbon C β
OE:	Oxygen O ϵ
OH:	Oxygen O η
OD:	Oxygen O δ
O:	Backbone Oxygen
NH:	Nitrogen N η
NE:	Nitrogen N ϵ
N:	Backbone Nitrogen
ND:	Nitrogen N δ

The principal IGF/IGFBP interaction, shown in the example of IGF-I mini-IGFBP-5 interaction, is a hydrophobic sandwich that consists of interlaced protruding side chains of IGF-I and solvent exposed hydrophobic side chains of the mini-IGFBP-5 (Figures 1 to 4). The side-chains of IGF-I Phe 16, Leu 54 and also Glu 3, are inserted deep into a cleft on the mini-IGFBP-5 (Figures 1 to 4). This cleft is formed by side chains of Arg 53, Arg 59 on the solvent exposed side of the molecule and by Val 49, Leu 70, Leu 74 on the opposite inner side, with a base formed by residues Cys 60 and Leu 61. Phe 16 makes direct contacts with the backbone and side chain of Val 49, and with Cys 60 of mini-IGFBP-5. The hydrophobic cluster is closed on the solvent side by side chains of Glu 3 and Glu 9 of IGF-I and His 71 and Tyr 50 of mini-IGFBP-5. These residues form a network of hydrogen bonds; in addition Arg 59 of mini-IGFBP-5 makes hydrogen bonds with Glu 58 (Figures 2 to 4).

Arg 53 and Arg 59 of mini-IGFBP-5 isolate the hydrophobic sandwich from the solvent close to the C-terminus. In the full length IGFBP-5, the segment corresponding to the C-terminus of mini-IGFBP-5 is followed by nine hydrophilic residues and then by at least 30 residues of mixed types. Thus, the conformations seen in the structure of the complex near the C-terminus of mini-IGFBP-5 are likely to be preserved in the complex of IGF-I with the full length-IGFBP-5. The mini-IGFBP-5 domain begins preferably at residue 40 of full length IGFBP-5.

The hydrophobic residues Val 49, Leu 70 and Leu 73 of IGFBP-5 are crucial for binding to IGFs. Since these residues are highly conserved among all IGFBPs, these hydrophobic interactions dominate the IGF binding properties of all IGFBPs.

The increased inhibitory potency of the mutant IGFBPs and fragments thereof results in the inhibition of the binding to and autophosphorylation of the IGF-IR (as described by Kalus, W., et al., in EMBO J. 17 (1998) 6558-6572) at significantly lower concentrations than observed for the wildtype proteins and the corresponding fragments. The higher potency of the mutant IGFBPs furthermore can be shown by the inhibition of the growth of tumor cells in vitro and in vivo. The growth of several tumor cell lines is known to be significantly reduced by IGFBPs. IGFBP-1 for example inhibits the growth of MCF-7 and MDA-MB-435A cells in vitro and the growth of tumors formed MDA-MB-231 cells in vivo in mice

(van den Berg, C.L., et al., Eur. J. Cancer 33 (1997) 1108-1113). IGFBP mutants with increased affinity inhibit the growth of these tumor cells at lower concentrations than the wild type proteins.

- 5 The following mutations of IGFBPs are preferred for enhancing binding affinity to IGF (numbering according to IGF-BP5 as aligned in Fig. 1) (standard one-letter abbreviation for amino acids used):

Table 1:
IGFBP-1

Amino acid No.	Original amino acid	Preferred mutations ¹⁾
48	V	L, <u>I</u> ,M,F,Y,W
49	A	<u>Y</u> ,R,K
52	R	W, <u>Y</u> ,M,F,H
60	R	Y,W,F
69	L	Y,W,M,I,F
72	L	I,Y,W,M, <u>F</u>
73	T	V,L,Y,W,M,I,F
74	R	H,D
82	E	R,K,H,N,Q,S,T,A,G

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Table 2:
IGFBP-2

Amino acid No.	Original amino acid	Preferred mutations ¹⁾
64	V	L, <u>I</u> ,M,F,Y,W
65	Y	R, <u>K</u>
68	R	W, <u>Y</u> ,M,F,H
76	Y	W,F
85	L	Y,W,M,I,F
86	Q	T,S,R,K,N, <u>H</u> ,Y,C
88	L	I, <u>Y</u> ,W,M,F
89	V	L,I,Y,W,M,F
90	M	H,D

Table 3:
IGFBP-3

Amino acid No.	Original amino acid	Preferred mutations ¹⁾
56	I	<u>L</u> ,V,M,F,Y,W
57	Y	R, <u>K</u>
60	R	W, <u>Y</u> ,M,F,H
68	Q	L,Y,W,F
75	R	Q
77	L	Y,W,M,I,F
78	Q	T,S,R,K,N, <u>H</u> ,Y,C
80	L	I,Y,W, <u>M</u> ,F
81	L	Y,W,M,I,F

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Table 4:
IGFBP-4

Amino acid No.	Original amino acid	Preferred mutations ¹⁾
48	V	L, <u>I</u> ,M,F,Y,W
49	Y	R, <u>K</u>
52	R	W, <u>Y</u> ,M,F,H
60	Y	W,F
67	K	Q
69	L	Y,W,M,I,F
72	L	I, <u>Y</u> ,W,M,F
73	M	Y,W,I,F
74	H	D

Table 5:
IGFBP-5

Amino acid No.	Original amino acid	Preferred mutations ¹⁾
49	V	L, <u>I</u> ,M,F,Y,W
50	Y	R, <u>K</u>
53	R	W, <u>Y</u> ,M,F,H
61	L	Y,W,F
68	K	Q
70	L	Y,W,M,I,F
73	L	I,Y, <u>W</u> ,M,F
74	L	Y,W,M,I,F
75	H	D
83	E	R,K,H,N,Q,S,T,A,G

Table 6:
IGFBP-6

Amino acid No.	Original amino acid	Preferred mutations ¹⁾
49	V	L, I ,M,F,Y,W
50	Y	R, K
53	N	R,W, Y ,M,F,H
61	H	L,Y,W,F
68	A	K,Q
70	L	Y,W,M,I,F
71	R	T,S, H ,K,N,Q,Y,C
73	L	I,Y, W ,M,I,F
74	L	Y,W,M,I,F
75	L	H,D

1) Amino acids are given in the standard one-letter amino acid code and are to be understood as alternative amino acid exchanges (or). For instance, the last line of Table 6 means that amino acid residue 75 of IGFBP-6, which is leucine (L), can preferably be modified to be either histidine (H) or aspartic acid (D). Table 6 is additionally to be interpreted such that amino acids 49, 50, 53, 61, 68, 70, 73, 74 and/or 75 can be exchanged in order to improve affinity. Especially preferred are IGFBP mutants with single point mutations. Most preferred are IGFBP mutants having a single point mutation from the bold face residues. This applies correspondingly to the other tables.

Table 7:
Sequence alignment
showing corresponding amino acids of IGFBP-1 to -6

Amino Acid No.					
IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6
48	64	56	48	49	49
49	65	57	49	50	50
52	68	60	52	53	53
60	76	68	60	61	61
67	83	75	67	68	68
69	85	77	69	70	70
70	86	78	70	71	71
72	88	80	72	73	73
73	89	81	73	74	74
74	90	82	74	75	75

The presented structure enables in silico screens for small IGFBP ligand inhibitors with the potential to release "free" bioactive IGF. Displacement of IGF from their binding proteins are therapeutically useful in treating a variety of potential indications, including short stature, renal failure, type I and type II diabetes, stroke and other neuro-degenerative diseases.

The compounds and IGFBP mutants of the present invention can be formulated according to methods for the preparation of compositions, preferably pharmaceutical compositions, which methods are known to the person skilled in the art. Preferably, such a compound and IGFBP mutant is combined in a mixture with a pharmaceutically acceptable carrier. Such acceptable carriers are described in, for example, Remington's Pharmaceutical Sciences, 18th ed., 1990, Mack Publishing Company, edited by Oslo et al. (e.g. pp. 1435-1712). Typical compositions contain an effective amount of a non-proteinaceous compound or IGFBP mutant according to the invention, for example from about 1 to 10 mg/ml, together with a suitable amount of a carrier. The compounds and IGFBP mutants may be administered preferably parenterally.

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The invention further provides pharmaceutical compositions containing a non-proteinaceous compound or IGFBP mutant according to the invention. Such pharmaceutical compositions contain an effective amount of a compound and IGFBP mutant of the invention, together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer contents (e.g., acetate, phosphate, phosphate-buffered saline), pH and ionic strength, additives such as detergents and solubilizing agents (e.g., Tween[®]80, polysorbate, Pluronic[®]F68), antioxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (Timersol[®], benzyl alcohol) and bulking substances (e.g., saccharose, mannitol).

Compositions and pharmaceutical compositions according to the invention are manufactured in that the substances in pure lyophilized form are dissolved at a concentration up to from 1 to 20 mg/l in PBS or physiological sodium chloride solution at a neutral pH value. For better solubility it is preferred to add a detergent.

Typically, in a standard cancer treatment regimen, patients are treated with dosages in the range of between 0.5 to 10 mg substance/kg weight per day.

The following examples, references, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Description of the Figures

Figure 1A: Sequence alignment of IGF-I and IGF-II. Bold underlined residues of IGF-I make contacts with mini-IGFBP5. Residues responsible for binding to the IGF-I receptor (IGF-IR) are marked with an asterisk above the sequence.

Figure 1B: Multiple sequence alignment of the N-terminal domains of human IGF-BPs 1-6. The mini-BP construct, numbered according to BP5 numbering, is marked above the aligned residues with „m“, including GS which indicate additional

residues from the cloning vector. (After position 81, mini-BP5 was disordered in the X-ray structure; this is indicated with italics.) BP5 residues that interact with IGF-I are shown underlined and in bold face. The degree of conservation of the residues is marked under the alignment with * for strict conservation, : for strict conservation of residue type, and . for relatively high conservation. The consensus sequence uses the following code to depict level of strict conservation: o alcohol, l aliphatic, a aromatic, c charged, h hydrophobic, - negative, p polar, + positive, s small, u tiny, t turnlike).

Figure 2: The overall structure of the IGF-I (tube model) mini-IGFBP5 (molecular surface) complex. Side chains plotted show the IGF residues in contact with BP5. Particularly important is Phe16, seen filling a hydrophobic depression on the BP5 surface.

Figure 3: Similar to Figure 2, whereby the IGF is depicted with its molecular surface and BP5 is depicted as a tube model. Side chains of BP5 responsible for binding to IGF are also depicted. The surface of IGF Phe16 is prominent, as is the relatively flat hydrophobic IGF surface contributing to the interface.

Figures 4A and 4B:

Summary of IGF-BP5 and IGF-I contacts. Interactions contributing to the binding affinity consist of hydrophobic interactions (a) (involving especially residues Leucines 70, 73, and 74 of BP5 and Phe16 of IGF-I) and also polar interactions (b). Enhancement of BP-IGF binding relies especially on the enhancement of hydrophobic interactions, either by increasing the intermolecular contact surface with these or with additional residues, or by the introduction of further polar contacts.

(A) Packing contacts between IGFBP-5 and IGF-I. Contacts are denoted according to nearest distances, whereby the closest contacts include polar interactions.

(B) Polar contacts between IGFBP-5 and IGF-I. Abbreviations denote hydrogen bonds (HB), CH-O hydrogen bonds (CHB), salt

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bridge (SB), and side chain (SC) or main chain (MC) interactions.

Figure 5: Atomic coordinates of IGF-I in the complex with mini-IGFBP-5.

5 **Figure 6:** Atomic coordinates of mini-IGFBP-5 in the complex with IGF-I.

Figure 7: Binding of radioactive J-125 IGF-I to NIH 3T3 cells expressing the IGF-IR in the absence and in the presence of IGFBP-5 and compounds potentially interfering with complex formation between IGF-I and IGFBP-5

10 **Figure 8:** IGF-I induced autophosphorylation of the IGF-IR expressed by NIH 3T3 cells in the absence and in the presence of IGFBP-5 and compounds potentially interfering with complex formation between IGF-I and IGFBP-5

Sequence Listing

15

SEQ ID NO:1 Primer FBP5LY.

SEQ ID NO:2 Primer RBP5LY.

SEQ ID NO:3 Primer FBP5LM.

SEQ ID NO:4 Primer RBP5LM.

20 SEQ ID NO:5 Primer IBP4NdeI.

SEQ ID NO:6 Primer IBP4BamHI.

SEQ ID NO:7 Peptide GSALA.

SEQ ID NO:8 Peptide GSHMDEAIH.

Example 1

Crystallization, data collection and derivatization

Mini-IGFBP-5 was produced as described by Kalus, W., et al., in EMBO J. 17 (1998) 6558-6572 and in Example 6, and IGF-I was obtained from OvoPepi, Australia. Crystallization was successful with 10% Jeffamine M-600, 0.1 M sodium citrate, 0.01 M ferric chloride, pH 5.6. Within 11 days, crystals appeared at 4 °C, growing to a final size of about 0.3 x 0.2 x 0.2 mm³. They belong to the cubic space group P213 and have unit cell dimensions a, b, c = 74.385 Å, with one complex molecule per asymmetric unit. Soaking in precipitation buffer with heavy atom compounds yielded a derivative K₂PtCl₄ (2.7 mM, 3 d) which was interpretable. All diffraction data were collected using a 300 mm MAR Research (Hamburg, Germany) image plate detector mounted on a Rigaku (Tokyo, Japan) RU300 rotating anode X-ray generator with graphite monochromatized CuK α radiation. All image plate data were processed with MOSFLM (Leslie, A.G.W., Molecular Data in Processing, in: Moras, D., Podjarny, A.D., and Thierry, J.C. (eds.), Crystallographic Computing 5 (1991), Oxford University Press, Oxford, UK, pp. 50-61) and the CCP4 program suite (Collaborative Computational Project, Number 4 1994).

Example 2

Phase calculation, model building and refinement

The structure of the IGF/mini-IGFBP-5 complex was solved by the single isomorphous replacement (s.i.r.) method using one heavy atom derivative described above. Derivative data was analyzed with the native data set, first using isomorphous difference Patterson maps and employing the Patterson vector superposition methods implemented in SHELX-97 (Sheldrick, G., Tutorial on automated Patterson interpretation to find heavy atoms, in: Moras, D., Podjarny, A.D., and Thierry, J.C. (eds.), Crystallographic Computing 5 (1991), Oxford University Press, Oxford, UK, pp. 145-157). The 2 heavy sites locations were confirmed by difference Fourier methods with appropriate initial single site s.i.r. phases using CCP4 programs. The refinement of heavy atom parameters and calculation of s.i.r. phases were done with SHARP (de la Fortelle, E., and de Briconne, G., Methods Enzymol. 276 (1997) 472-494). The final parameters are given in Table 8. The initial s.i.r. phases were improved with SOLOMON

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(Abrahams, J.P., and Leslie, A.G.W., *Acta. Cryst. D*52 (1996) 30-42) using an solvent fraction of 45%, resulting in a 2.1 Å electron density map that was interpretable. Refinement was performed by conjugate gradient and simulated annealing protocols as implemented in CNS 1.0 (Brünger, A.T., et al., *Acta Crystallogr. D*54 (1998) 905-921. All protocols included refinement of individual isotropic B-factors and using the amplitude based maximum likelihood target function. The R-factor dropped to 21.8 % (R_{free}= 26.2 %, resolution range 16.2 – 2.1 Å) for the final model including 47 water molecules. The water model was calculated using ARP and verified by visual inspection. The final refinement statistics are shown in Table 8.

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Table 8:
Statistics from the crystallographic analysis

	native	K ₂ PtCl ₄
Resolution (Å)	16.2 – 2.1	18.6 – 2.5
Measurements	45345	32833
Unique measurements	8035	4925
% Complete (last shell/Å)	99.3 (96.9/2.23 – 2.11)	99.9 (95.4/2.64-2.5)
R_{sym} (%) (last shell)	8.2 (44.8)	8.8 (49.5)
$R_{Cullis-iso}$	-	0.77
P_{iso}	-	1.48
Res. for phase calc. (Å)	-	18.6 – 2.5
Mean FOM	-	0.41

Refinement statistics:

Resolution range (Å)	16.2 – 2.1
reflections in working set	7522
reflections in test set	501
R_{cryst} (%)	21.8
R_{free} (%)	26.2
Protein atoms (non-H)	765
Solvent atoms (non-H)	47
Average B-factor (Å ²)	38.1
r.m.s. ΔB (2Å cutoff)	3.4
Deviations from ideality (r.m.s.):	
Bond lengths (Å)	0.013
Bond angles (°)	1.7

5

$$R_{sym} = \frac{\sum |I(h)_i - \langle I(h) \rangle|}{\sum \langle I(h) \rangle}$$

 $R_{Cullis-iso}$ = r.m.s. lack of closure / r.m.s isomorphous difference

 P_{iso} (Phasing power) = $\langle |F_H| \rangle$ / r.m.s. lack of closure for all reflections

Mean FOM = mean figure of merit

R_{crist} = Crystallographic R-factor for reflections used in refinement

R_{free} = Crystallographic R-factor for reflections not used in refinement

r.m.s. = Root mean square

Example 3

5 Determination of the binding affinity of IGFBP mutants

The IGF-binding properties of wildtype and mutant fragments and full-length IGFBPs were quantitatively analyzed by BIAcore biosensor measurements. BIAcore 2000, Sensor Chip SA and HBS were obtained from BIAcore AB (Uppsala, Sweden). All experiments were performed at 25°C and HBS (20 mM HEPES, 150 mM NaCl, 3 mM EDTA, pH 7.5) was used as a running buffer and for the dilution of ligands and analytes. Biotinylated IGF-I was immobilized at a concentration of 5 nM and 10 nM in HBS at a flow rate of 5 µl/min to the streptavidin coated sensor chip resulting in signals of 40 and 110 resonance units (RU). Biotinylated IGF-II was immobilized at a concentration of 5 nM in HBS resulting in a signal of 20 RU. An empty flow cell was used as control for unspecific binding and bulk effects. The low ligand concentration was necessary to limit mass transport limitations and rebinding. For the same reason all kinetic experiments were performed at the highest possible flow rate of 100 µl/min. Each protein (wildtype and mutant IGFBPs or fragments of these proteins) was injected at four concentrations (250, 50, 10, and 2 nM). Each sample was injected for 2 min followed by dissociation in buffer flow for 4 min. After the dissociation phase the sensor chip was regenerated by injection of 10 µl 100 mM HCl at a flow rate of 5 µl/min. The kinetic parameters were calculated using the BIAevaluation 3.0 software (BIAcore AB). After subtraction of the blank sensorgram the kinetic rate constants were calculated from a general fit of an overlay of the sensorgrams of all concentration of one analyte using the method called "1:1 binding with mass transfer". IGF-I and IGF-II were biotinylated with a five-fold molar excess of D-biotinyl-ε-aminocaproic acid-N-hydroxysuccinimide ester using the reagents and the operation instructions of the Biotin Protein Labelling Kit (Roche Diagnostics GmbH, DE). After blocking with lysine, the reaction was dialyzed against 50 mM Na-phosphate, 50 mM NaCl, pH 7.5.

Example 4**Inhibition of IGF-I-induced IGF-IR phosphorylation by IGFBP mutants**

Confluent monolayers of NIH3T3 cells stably expressing human IGF-IR in 3.5 cm dishes were starved in DMEM containing 0.5% dialyzed fetal calf serum. After 48 h, cells were incubated without any hormone or with 5×10^{-9} M IGF-I or 1×10^{-8} M IGF-II; each sample was preincubated with increasing concentrations of different IGF-binding proteins or fragments thereof at room temperature for 1 h. After a 10 min stimulation at 37°C, the medium was removed and cells were lysed with 250 µl of lysing buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Nonidet P40, 1.5 mM MgCl₂, 1 mM EGTA (ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid, Aldrich, USA), 10 mM sodium orthovanadate, and protease inhibitor cocktail Complete (Roche Diagnostics GmbH, DE) for 10 min on ice. Subsequently, cells were scraped off the plate and the insoluble material was separated by centrifugation for 20 min at 4°C. The protein concentration of the supernatant was determined using the BCA kit from Pierce, Rockford, USA according to the manufacturer's instructions. Equal protein concentration was incubated with the SDS sample buffer (63 mM Tris-HCl, pH 6.8, 3% SDS, 10% glycerol, 0.05% bromphenolblue, 100 mM DTT), boiled for 5 min and loaded on a 7.5% SDS polyacrylamide gel. After electrophoresis the proteins were transferred on a nitrocellulose membrane which first was blocked for 1 h with the 3 % BSA containing PBST (phosphate buffered saline-Tween®), then overnight incubated with 1 µg/ml monoclonal anti-phosphotyrosine antibody 3-365-10 (Roche Diagnostics GmbH, DE) in PBST that contained 3% BSA. Unbound antibody was removed by extensive washing. The blot was then incubated with 1:10000 diluted anti-mouse IgG-specific antibody conjugated with horse raddish peroxidase (Roche Diagnostics GmbH, DE). The immunoblot was developed using the ECL kit from Amersham and the concentration of IGFBP which results in 50 % inhibition of the IGF-I receptor phosphorylation was determined.

Example 5**Determination of the inhibition of tumor cell growth by IGFBP mutants**

MCF-7 cells (from ATCC, American type Culture Collection, Rockville, Maryland, U.S.A., HTB22) were used to investigate the inhibitory effect of IGFBP mutants on tumor cells. 1000 MCF-7 cells were seeded per well in medium containing 2.5 %

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FBS (fetal bovine serum). The cells were cultured in the presence of various concentrations of IGFBPs for 48 h. The percentage of surviving cells was determined by MTT ((3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide) assay and the concentration of binding protein which results in reduction of cell survival by 50 % was determined.

Example 6

Mutagenesis, expression and purification of mini-IGFBP-5s and subcloning of IGFBP-4 into Pet-28a (+)

6.1 Buffers and media

10 Cell growth media:

LB-medium	per 1 liter: peptone 10 g, yeast extract 5 g, NaCl 10 g, adjusted to pH 7.
LB-agar	per 1 liter: peptone 10 g, yeast extract 5 g, NaCl 10 g, bacto agar 15 g, adjusted to pH 7.
Minimal medium	per 1 liter: 0.5 g NaCl, 1 g citric acid monohydrate, 36 mg ferrous citrate (pre-dissolved in conc. HCl), 4.02 g KH ₂ PO ₄ , 7.82 g K ₂ HPO ₄ , 1g ¹⁵ N-NH ₄ Cl, 1.3 ml trace elements solution (per liter of the stock solution: 2.5 g H ₃ BO ₃ , 2.0 g CoCl ₂ , 1.13 g CuCl ₂ , 9.8 g MnCl ₂ , 2.0 g Na ₂ MoO ₄), 1 ml Zn-EDTA solution (per ml of the stock solution: 5 mg EDTA, 8.4 mg zinc acetate), adjusted to pH 7, autoclaved. Added afterwards: 25 ml autoclaved 20% (w/v) glucose, 560 µl sterile filtered 1% (w/v) thiamine, 2ml 1M MgSO ₄ .

Antibiotic stocks:

Ampicillin	50 mg/ml in dist. water, 0.22 µm filtrated, stored at -20°C.
Kanamycin	25 mg/ml in dist. water, 0.22 µm filtrated, stored at -20°C.
Chloramphenicol	35 mg/ml in 96 % ethanol, stored at -20 °C.

Agarose-gel electrophoresis:

	TAE-buffer (50x)	2 M Tris-HCl (pH 8.0), 2 M glacial acetic acid and 50 mM EDTA.
5	Loading buffer (3x)	0.13 % bromophenol blue, 0.13 % xylene cyanol, 30 % glycerol.
	Et-Br-solution	10 mg/ml ethidiumbromide in dd H ₂ O.

SDS-PAGE:

10	Sample buffer (5x)	125 mM Tris-HCl (pH 6.8), 10 % SDS, 760 mM 2-mercaptoethanol, 0.13 % bromophenol blue, 50 % glycerol and 2 mM EDTA.
	Staining solution	0.125 % CBB-R250 in 500 ml 96 % ethanol and 500 ml 10 % acetic acid.
15	Distaining solution	96 % ethanol, 10 % acetic acid and dest. H ₂ O in 4:3:3 proportion.

Tricine gels:

20	Cathode (top)	
	running buffer (10x)	1 M Tris-HCl (pH 8.25), 1 M Tricine and 1 % SDS.
	Anode (bottom)	
	running buffer (10x)	2 M Tris-HCl (pH 8.9).
25	Separation buffer	3 M Tris-HCl (pH 8.9) and 0.3 % SDS.
	Stacking buffer	1 M Tris-HCl (pH 6.8) and 0.3 % SDS.
	Separation acrylamide	48 % (w/v) acrylamide, 1.5 % (w/v) N,N'-methylene-bis-acrylamide.
	Stacking acrylamide	30 % (w/v) acrylamide, 0.8 % (w/v) N,N'-methylene-bis-acrylamide.
30	APS	10 % ammonium persulphate in dd H ₂ O.
	Separation gel (main)	for 2 70x80x0.75 mm mini-gels: 1.675 ml H ₂ O, 2.5 ml separation buffer, 2.5 ml separation acrylamide, 0.8 ml glycerol, 25 µl APS and 2.5 µl TEMED.

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	Separation gel (intermediate)	1.725 ml H ₂ O, 1.25 ml separation buffer, 0.75 ml separation acrylamide, 12.5 µl APS and 1.25 µl TEMED.
5	Stacking gel	2.575 ml H ₂ O, 0.475 ml stacking buffer, 0.625 ml stacking acrylamide, 12.5 µl 0.5 M EDTA (pH 8.0), 37.5 µl APS and 1.9 µl TEMED.
	Protein purification:	
10	Buffer A	6 M guanidinium-HCl, 100 mM NaH ₂ PO ₄ , 10 mM Tris and 10 mM 2-mercaptoethanol, pH 8.0.
	Buffer B	6 M guanidinium-HCl, 100 mM NaH ₂ PO ₄ , 10 mM Tris and 10 mM 2-mercaptoethanol, pH 6.5
15	Buffer C	6 M guanidinium-HCl, 100 mM Na-acetate and 10 mM 2-mercaptoethanol, pH 4.0.
	Buffer D	6 M guanidinium-HCl, pH 3.0.
	Buffer E	200 mM arginine, 1 mM EDTA, 100 mM Tris-HCl, 2 mM reduced glutathione, 2 mM oxidised glutathione, pH 8.4.
20	PB(0)	10 mM Na ₂ HPO ₄ , 1.8 mM KH ₂ PO ₄ and 0.05 % NaN ₃ , pH 7.2.
	PB(1000)	10 mM Na ₂ HPO ₄ , 1.8 mM KH ₂ PO ₄ , 0.05 % NaN ₃ and 1 M NaCl, pH 7.2.
	PBS	140 mM NaCl, 27 mM KCl, 10 mM Na ₂ HPO ₄ , 1.8 mM KH ₂ PO ₄ and 0.05% NaN ₃ .
25	Thrombin cleavage buffer	60 mM NaCl, 60 mM KCl, 2.5 mM CaCl ₂ , 50 mM Tris, pH 8.0.

6.2 Cloning of mini-IGFBP-5

- 30 Mini-IGFBP-5 (residues 40-92 of IGFBP-5) was subcloned from a vector containing the complete sequence of IGFBP-5 into the BamHI and PstI restriction sites of the pQE30-vector (Qiagen, Hilden, Germany). Restriction sites, a stop codon and 21 bases encoding an N-terminal thrombin cleavage site were introduced by means of PCR (Kalus, W., et al., EMBO J. 17 (1998) 6558-6572).

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6.3 Mutagenesis of mini-IGFBP-5

For introduction of mutations leading to substitution of Leu₆₁ by Tyr and Leu₇₄ by Met, in vitro mutagenesis was performed using QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, Canada). Two sets of the following mutagenic oligonucleotide primers were designed for amplification of plasmid DNA and introduction of the desired point mutations:

FBP5LY: 5'-G GGG CTG CGC TGC TAC CCC CGG CAG GAC G-3';

(SEQ ID NO:1)

RBP5LY: 5'-C GTC CTG CCG GGG GTA GCA GCG CAG CCC C-3';

(SEQ ID NO:2)

FBP5LM: 5'-CG CTG CAC GCC CTG ATG CAC GGC CGC GGG G-3';

(SEQ ID NO:3)

RBP5LM: 5'-C CCC GCG GCC GTG CAT CAG GGC GTG CAG CG-3'

(SEQ ID NO:4).

The changed codons (CTC into TAC in L₆₁Y mutant and CTG into ATG in L₇₄M mutant) are presented in bold. Degenerated bases are underlined.

The reactions were set up according to the instructions found in the mutagenesis kit manual. The PCR mixtures (50 µl) contained app 50 ng of the template (pQE30 (mini-IGFBP-5), prepared by means of mini prep spin columns kit, Qiagen) and 125 ng of each of the two oligonucleotide primers. Cycling parameters for the reactions were as follows: 30 seconds at 95°C followed by 13 cycles of 95°C for 30 seconds, 55°C for 1 minute and 68°C for 7.5 min. The DpnI digestion and XL1-Blue supercompetent cells transformation was carried out strictly according to the supplier's guidelines.

Two clones of each mutant were subjected to verification by automated double stranded sequencing, which proved the existence of the expected substitutions in all 4 cases.

6.4 Expression of the mutant mini-IGFBP-5s

Electrocompetent cells BL21 were transformed with the construct carrying the mutation. From a fresh plate, a 3-ml LB culture was started and grown overday (6-7 h) in the presence of 300 µg ampicillin per ml at 37°C. From this culture 50 µl were used to inoculate 20 ml of MM. This culture was grown overnight (9-11h). 1 l culture was inoculated in 1:50 proportion. Expression of the protein was induced at OD₆₀₀ ≅ 0.8 by addition of IPTG (1 mM final concentration). Cells were harvested after 3 h (6000 xG, 20 min at 4°C).

6.5 Purification of mini-IGFBP-5

Harvested cells were resuspended in buffer A (30 ml of the buffer was used to resuspend cells from 1 l culture) and incubated at room temperature with vigorous shaking (280 RMP) for 1 h to overnight. The cells were opened by sonification (macrotip, 50 % duty cycle, output control 70, 2x4 min). The cell extract was then centrifuged to pellet cell debris (65 000 xG, 1h at room temp.). The pH of the supernatant was adjusted to the value of app. 8.0. The supernatant was then mixed with pre-equilibrated with buffer A Ni-NTA Superflow matrix (Qiagen) , incubated with agitation for 1 h to overnight and then loaded onto an empty column (3 ml bed volume for 1 l culture). The column was washed with buffer A followed by buffer B until a stable UV-absorption base line. Bound proteins were fractionated with 100 ml pH gradient of buffer B and C. Collected fractions were analysed by tricine gel electrophoresis (prior electrophoresis, the proteins were precipitated with 5 % (w/v) TCA). Fractions containing mini-IGFBP-5 were pooled, concentrated on Amicon YM3 to 2-4 ml, and dialysed against 2 l of buffer D overnight (100 µl excess of 2-mercaptoethanol was added to the sample prior dialysis).

To promote refolding, the dialysed sample was diluted in 100 µl portions into freshly prepared, ice-cold buffer E, with vigorous stirring (in proportion 1 ml sample per 50 ml of buffer E), and left at 4°C for 2-3 days with stirring.

The sample was concentrated on Amicon YM3 to 15-25 ml, centrifuged to get rid of a precipitated material, and dialysed overnight into 4 l of buffer PB containing 30 mM NaCl.

The solution was subsequently loaded onto pre-equilibrated with buffer PB (0) MonoS 5/5 HR cation-exchanger column (app. 1 ml) (Amersham Pharmacia, Uppsala, Sweden) at a flow rate of 1 ml/min. The column was washed with buffer PB (0). Proteins were eluted by 45 ml linear gradient of 0-70 % NaCl, 1 ml fractions
5 were collected.

The fractions containing mini-IGFBP-5 (as determined on the basis of tricine gel electrophoresis) were pooled, concentrated to 2-3 ml and loaded onto a pre-equilibrated with PBS Superdex 75 HiLoad 26/60 (app. 320 ml) gel-filtration column (Pharmacia) at a flow rate of 0.6 ml/min. Mini-IGFBP-5 was eluted as a
10 symmetrical, single pick. Fractions containing the protein were pooled and concentrated on centricon YM3.

6.6 Subcloning into pET-28a (+)

The reason for overall low expression of the proteins from the pQE30 might be the fact that this vector is not well optimised for expression in *E. coli*. For instance, the
15 vector-encoded sequences contain a cluster of 3 rare codons just downstream from the initiator codon AUG (namely, AGA, GGA and TCG, encoding Arg, Gly and Ser, respectively). The number of studies has indicated that excessive rare codon usage in a target gene may be a cause for low level expression. The impact seems to be most severe when multiple rare codons occur near the amino terminus and when
20 they appear consecutively. Especially presence of the Arg codons AGG and AGA can have severe effects on the level of protein production. The system seems to be also not well repressed (no extra copies of a gene encoding Lac repressor), and the leaky expression may cause the observed plasmid instability. The vector carries not very efficient selective marker, Amp^R gene (bla), what makes possible rapid over-
25 growing of a culture at a certain stage by cells lacking the unstable plasmid. The vector pET-28a (+) (Novagen) was then chosen as an alternative for pQE30. The plasmid is well optimised for expression of genes in *E. coli*, carries a strong selective marker (Kan^R) and is stable due to high level of repression of the target gene expression in the absence of IPTG (in a non-DE3 lysogenic strain even in the
30 presence of the inducer).

To subclone mini-IGFBP-5 wild type, L₆₁Y and L₇₄M from pQE30 to pET-28a, the relevant fragments were excised from the vector with BamHI and HindIII (HindIII

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cleavage site exists in pQE30 downstream from PstI site). The excision was performed as double-digestion. Digested pET vector was 5'-dephosphorylated. Reaction mixtures were electrophorized and bands corresponding to app. 200 bp fragments excised from pQE30 (mini-IGFBP-5 wt, L₆₁Y and L₇₄M) and app 5000 bp fragment of pET-28a were cut from 1 % agarose gel and purified (gel extraction kit, Qiagen). The fragments were ligated (Ligation kit, Fermentas) and XL-1 Blue Supercompetent cells were transformed with the ligation mixture.

Restriction assay carried out subsequently on isolated plasmid DNA revealed presence of fragments of expected size (restriction enzymes NcoI and PstI were used, double digestion was performed. PstI restriction site was introduced into the pET vector together with the fragment encoding mini-IGFBP-5).

Pilot-scale expression and purification experiment showed that expression of the protein of interest (mini-IGFBP-5 L₆₁Y in this case) is higher than the expression of the wild-type protein when pQE30 vector was used.

The proteins are expressed as double-fusions: they carry His-tag followed by T7-tag. It is possible to remove both tags by thrombin cleavage. Mini-IGFBP-5 after cleavage by thrombin comprises the following N-terminal amino acid sequence: GSALA (SEQ ID NO:7) (N-terminus of mini-IGFBP-5 starting from aa 40 with to additional aa from cloning with thrombin cleavage site). Vector-derived amino acids are underlined.

6.7 Subcloning of IGFBP4 from pKK177-3HB to pET-28a(+)

For subcloning of IGFBP4-2 into the NdeI and BamHI restriction sites of the pET-28a vector in-frame to a His-tag, following oligonucleotides were designed for amplification of DNA by PCR:

IBP4NdeI: 5'-CGG AGG AAA AAC ATA TGG ATG AAG C-3'
(SEQ ID NO:5)
IBP4BamHI: 5'-GCC AAG CTT GGA TCC AGG TCG AC-3'
(SEQ ID NO:6)

The restriction sites recognized by NdeI and BamHI are presented in bold. Degenerated bases are underlined.

The PCR mixture (50 µl) contained 124 ng of mixture of pKK177-3HB and Pfdx500 repressor plasmid, 130 ng of each of the primers, 1 µl dNTP mix and 2.5 U Pfu Turbo DNA polymerase (Stratagene). After initial step of 30 sec. At 95°C, the reaction was cycled 30x at 95°C for 30 seconds, 55°C for 1 min and 68°C for 2 min. The product of PCR was purified (PCR purification kit, Qiagen), double-digested and electrophorised. The bands corresponding to cleaved pET-28a and PCR product were excised from the gel and purified.

XL-1 Blue Supercompetent cells were transformed with the ligation mixture.

IGFBP4-2 is expressed as a N-terminal His-tag fusion protein. After thrombin cleavage, the protein comprises the following amino acid sequence: GSHMDEAIH... (SEQ ID NO:8). Vector derived amino acids are underlined.

The same purification routine will be used for His-tagged IGFBP-4 as for mini-IGFBP-5.

Example 7

Identification of chemical non-proteinaceous compounds binding to IGFBP-5 or IGF-I by using the coordinates of the crystal structure of the complex of both molecules

FlexX version 1.9.0 was used to screen a substance library of ca. 90,000 compounds in an ACD (Available Chemicals Directory; ACD-3D 2000), choosing compounds with a molecular weight of less than 550 Daltons and containing at least one of the atoms {N, O, F, or S}. Docking searches were conducted on both molecular surfaces of the IGFBP-5 interface. Top scoring hits as judged by the FlexX standard scoring function and the proximity to binding site protein atoms were selected and tested for activity.

The top scoring compounds selected according to these these criteria for release of IGF-I from IGFBP-5 were:

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- Compound 1: N1-(3,4-Dichlorophenyl)-2-[2-[5-(3,5-dichlorophenyl)-2H-1,2,3,-tetraazol-2YL]A (MF: C₁₆H₁₁Cl₄N₇O₅; MW: 491,1890 Da)
- Compound 2: F-MOC-Tyr(PO₃H₂)-OH (C₂₄H₂₂NO₈P; MW: 483.4110)
- 5 Compound 2A: N α -FMOC-O-tert-butyl-L-tyrosine
- Compound 2B: N α -FMOC-L-phenylalanine
- Compound 2C: N α -FMOC-N-BOC-L-tryptophan
- Compound 2D: N α -FMOC-L-leucine
- 10 Compound 3: 4-(2,5-Dichlorophenylazo)-4'-fluorosulfonyl-1-hydroxy-2-naphthanilide (MF: C₂₃H₁₄Cl₂FN₃O₄S; MW: 518.3510)
- Compound 4B: 5-Amino-2[(4-amino-2-carboxyphenyl)thio]benzoic acid (C₁₄H₁₂N₂O₄S; MW 304.3250)
- Compound 4C: 5-Amino-2[(2-carboxyphenyl)thio]benzoic acid (C₁₄H₁₁NO₄S; MW 289.3100)
- 15 **Example 8**
- Release of IGF-I from the complex with IGFBP-5 by selected compounds measured by IGF-I binding to IGF-IR expressing cells
- 20 Kalus, W., et al., in EMBO J. 17 (1998) 6558-6572, describe the inhibition of the binding of IGF-I to IGF-IR expressing NIH 3T3 cells by formation of an inhibitory complex. This assay was used to investigate the release of IGF-I from the inhibitory complex with IGFBP-5.
- 25 NIH 3T3 cells stably expressing human IGF-IR were grown in culture dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. Cells were washed carefully with PBS and incubated with 5 ml of 50 mM EDTA in PBS for 45 min. Cells were removed from the plate, washed once with PBS and once with binding buffer (100 mM HEPES pH 7.6, 120 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 1 mM EDTA, 10 mM glucose, 15 mM sodium acetate, 1% dialysed BSA), and resuspended in binding buffer to determine the cell number. 5 pM ¹²⁵I-IGF-I (Amersham) was preincubated with either 10 or 100 nM IGFBP-5 alone or in

combination with 33 μ M of the different compounds 1,2,3,4B and 4C at 4°C for 1 h. Then 400 μ l of the cell suspension corresponding to 2×10^5 cells were added to give a total volume of 500 μ l. After 12 h incubation at 4°C, cells were washed with binding buffer (at 4°C). Free hormone was removed by repeated centrifugation and resuspension in the binding buffer. The 125 I radioactivity bound to the cells was determined in a gamma-counter.

As shown in figure 7 the labeled IGF-I binds to NIH 3T3 cells in the absence of IGFBP-5 and cell binding is inhibited by the addition of IGFBP-5. Preincubation of the complex of IGFBP-5 and IGF-I with the selected compounds results in release of IGF-I from the complex by compound 3 and consequently binding of IGF-I to the IGF-IR expressing cells.

Example 9

Release of IGF-I from the complex with IGFBP-5 by selected compounds measured by IGF-IR activation

Kalus, W., et al., in EMBO J. 17 (1998) 6558-6572, describe the inhibition of the activation and autophosphorylation of the IGF-IR by IGF-I in the presence of IGFBP-5. This assay was used to further investigate the release of IGF-I from the inhibitory complex with IGFBP-5 by compound 3. Binding of compound 3 to IGFBP-5 and dissociation of the complex of the binding protein with IGF-I should result in an activation and autophosphorylation of the IGF-IR in the presence of IGFBP-5.

Confluent monolayers of the NIH 3T3 cells stably expressing human IGF-IR in 3.5 cm dishes were starved in DMEM containing 0.5% dialysed fetal calf serum. After 48 h, cells were incubated without any hormone or with 10 nM IGF-I. Samples were preincubated with 100 nM IGFBP-5 and increasing concentrations of compound 3 from 0 to 50 μ M at room temperature for 1 h. After a 10 min stimulation at 37°C, the medium was removed and cells were lysed with 250 μ l of lysing buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% NP-40, 1.5 mM $MgCl_2$, 1 mM EGTA), 10 mM sodium orthovanadate, and protease inhibitor cocktail Complete (Roche Molecular Biochemicals) for 10 min on ice. Subsequently, cells were scraped off the plate and the insoluble material was separated by centrifugation for 20 min at 4°C. The protein concentration of the

supernatant was determined using the BCA kit from Pierce according to the manufacturer's instructions. Equal protein concentration was incubated with the SDS sample buffer (63 mM Tris-HCl pH 6.8, 3% SDS, 10% glycerol, 0.05% bromophenolblue, 100 mM DTT), boiled for 5 min and loaded on a 7.5% SDS-polyacrylamide gel. After electrophoresis the proteins were transferred on a nitrocellulose membrane which first was blocked for 1 h with the 3% BSA containing phosphate-buffered saline-Tween (PBST), then incubated overnight with 1 mg/ml monoclonal anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology), polyclonal anti-phospho-AKT antibody (New England Biolabs) or polyclonal anti-IGF-IR (C-20, Santa Cruz Biotechnology) in PBST that contained 3% BSA. Unbound antibody was removed by extensive washing. The blot was then incubated with 1:10 000 diluted anti-mouse IgG-specific antibody or 1:5000 diluted anti-rabbit specific antibody conjugated with horse radish peroxidase (both Roche Molecular Biochemicals). The immunoblot was developed using the ECL kit from Amersham.

As shown in Fig. 8 the autophosphorylation of IGF-IR by IGF-I is inhibited in the presence of IGFBP-5. The addition of compound 3 to the inactive complex of IGFBP-5 and IGF-I results in an increased autophosphorylation of the receptor at 50 μ M compound 3.

Example 10

Detection of ligand binding

Ligand binding was detected by acquiring ^{15}N -HSQC spectra. All NMR spectra were acquired at 300 K on Bruker DRX600 spectrometer. The samples for NMR spectroscopy were concentrated and dialyzed against PBS buffer. Typically, the sample concentration was varied from 0.3 to 1.0 mM. Before measuring, the sample was centrifuged in order to sediment aggregates and other macroscopic particles. 450 μ l of the protein solution were mixed with 50 μ l of D_2O (5-10%) and transferred to an NMR sample tube. The stock solutions of compounds were 100 mM either in water or in perdeuterated DMSO. pH was maintained constant during the whole titration. The binding was monitored by observation of the changes in the ^{15}N -HSQC spectrum. Dissociation constants were obtained by monitoring the chemical shift changes of the backbone amide of several amino acid residues (Table 9) as a function of ligand concentration. Data were fit using a single

binding site model. In the same way dissociation constants for derivatives of compound 2 are estimated (Table 10).

Table 9:

Dissociation constant calculations for compound 2 or DMSO binding to IGFBP-5
using data from distinct amino acid residues

residue	ligand in DMSO K_D [mM]	ligand in PBS K_D [mM]	DMSO K_D [mM]
Y50	1.58 ± 0.09	1.82 ± 0.95	648 ± 370
L73	1.31 ± 0.17	2.93 ± 1.41	541 ± 306
S85	1.38 ± 0.10	2.33 ± 0.94	650 ± 373
Y86	1.90 ± 0.17	1.72 ± 0.99	783 ± 498
R87	1.64 ± 0.12	2.36 ± 1.00	921 ± 662
K91	2.42 ± 0.18	2.12 ± 1.03	719 ± 434
average:	1.71 ± 0.37	2.21 ± 0.40	710 ± 120

Table 10:

Dissociation constants calculated for compound 2 and its derivatives binding to
IGFBP-5 using changes in chemical shift for the residue L81

compound	chemical name	K_D [mM]
2	N α -FMOC-O-phospho-L-tyrosine	2.78 ± 0.30
2A	N α -FMOC-O-tert-butyl-L-tyrosine	0.718 ± 0.079
2B	N α -FMOC-L-phenylalanine	1.075 ± 0.507
2C	N α -FMOC-N-BOC-L-tryptophan	0.0432 ± 0.0115
2D	N α -FMOC-L-leucine	1.088 ± 0.519

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Patent Claims

1. A crystal suitable for X-ray diffraction, comprising a complex of insulin-like growth factor I or II (IGF) and a polypeptide consisting of the amino acids 39-91 of IGFBP-1, the amino acids 55-107 of IGFBP-2, the amino acids 47-99 of IGFBP-3, the amino acids 39-91 of IGFBP-4, the amino acids 40-92 of IGFBP-5, or the amino acids 40-92 of IGFBP-6 or a fragment thereof consisting at least of the 9th to 12th cysteine of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, or IGFBP-5 or at least of the 7th to 10th cysteine of IGFBP-6, to form a complex which exhibits restricted conformation mobility.
2. A crystal of claim 1, which effectively diffracts X-ray for the determination of the atomic coordinates of the complex to a resolution of 1.5 to 3.5 Å.
3. A crystal of claim 1 or 2, wherein the crystal is arranged in the cubic space group P2₁3 having unit cell dimensions of 74.385 Å x 74.385 Å x 74.385 Å.
4. A method for producing a crystal suitable for X-ray diffraction, comprising
 - (a) contacting IGF with a polypeptide consisting of the amino acids 39-91 of IGFBP-1, the amino acids 55-107 of IGFBP-2, the amino acids 47-99 of IGFBP-3, the amino acids 39-91 of IGFBP-4, the amino acids 40-92 of IGFBP-5, or the amino acids 40-92 of IGFBP-6 or a fragment thereof consisting at least of the 9th to 12th cysteine of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, or IGFBP-5 or at least of the 7th to 10th cysteine of IGFBP-6, to form a complex which exhibits restricted conformation mobility, and
 - (b) obtaining a crystal from the complex so formed suitable for X-ray diffraction.
5. A method for the determination of the atomic coordinates of a crystal suitable for X-ray diffraction obtained by
 - (a) contacting IGF with a polypeptide consisting of the amino acids 39-91 of IGFBP-1, the amino acids 55-107 of IGFBP-2, the amino acids 47-99

- 5 of IGFBP-3, the amino acids 39-91 of IGFBP-4, the amino acids 40-92 of IGFBP-5, or the amino acids 40-92 of IGFBP-6 or a fragment thereof consisting at least of the 9th to 12th cysteine of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, or IGFBP-5 or at least of the 7th to 10th cysteine of IGFBP-6, to form a complex which exhibits restricted conformation mobility; and
- (b) obtaining a crystal from the complex so formed suitable for X-ray diffraction;
- (c) determining the atomic coordinates of said crystal.
- 10 6. A method for identifying a mutant of IGFBP (IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5 or IGFBP-6 or a mutant of a fragment thereof consisting at least of the 9th to 12th cysteine of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, or IGFBP-5 or at least of the 7th to 10th cysteine of IGFBP-6) having an enhanced binding affinity for IGF, comprising
- 15 (a) constructing a three-dimensional structure of the complex of IGF and a polypeptide consisting of the amino acids 39-91 of IGFBP-1, the amino acids 55-107 of IGFBP-2, the amino acids 47-99 of IGFBP-3, the amino acids 39-91 of IGFBP-4, the amino acids 40-92 of IGFBP-5, or the amino acids 40-92 of IGFBP-6 consisting at least of the 9th to 12th cysteine of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, or IGFBP-5 or at
- 20 least of the 7th to 10th cysteine of IGFBP-6, based on the atomic coordinates of a crystal consisting of IGF and said IGFBP or a fragment thereof;
- (b) employing said three-dimensional structure and modeling methods to
- 25 identify said mutant of an IGFBP in which a residue within a distance of 5 Å to a hydrophobic amino acid residue of IGF is modified in that the hydrophobic interaction between IGF and said mutant of IGFBP is enhanced;
- (c) producing said mutant;

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- (d) assaying said mutant to determine said enhanced binding affinity for IGF.
7. A method for identifying a mutant of IGFBP-5 with enhanced binding affinity for IGF-I, said method comprising
- 5 (a) constructing a three-dimensional structure of the complex of IGF and IGFBP-5 defined by the atomic coordinates shown in Figs. 5 and 6;
- (b) employing said three-dimensional structure and modeling methods to identify an amino acid residue in IGFBP-5 within a distance of 5 Å or shorter to an amino acid residue of IGFI, wherein said residue of
10 IGFBP-5 can be modified hydrophobically in that the hydrophobic interaction between IGF and IGFBP-5 is enhanced;
- (c) producing said mutant;
- (d) assaying said mutant to determine said enhanced binding affinity for IGF.
- 15 8. A mutant of IGFBP containing one or more of the mutations as depicted in Tables 1 to 6.
9. A mutant of IGFBP containing one or more mutations of amino acid residues 49, 70 and/or 73 corresponding to IGFBP-5 sequence alignment according to Tables 1 to 6.
- 20 10. A method for identifying a non-proteinaceous compound capable of binding to IGFBP, comprising
- (a) constructing a three-dimensional structure of a complex of insulin-like growth factor I or II and a polypeptide consisting of the amino acids
25 40-92 of insulin-like growth factor binding protein 5, amino acids 39-91 of IGFBP-1, amino acids 55-107 of IGFBP-2, amino acids 47-99 of IGFBP-3, amino acids 39-91 of IGFBP-4, amino acids 40-92 of IGFBP-5, amino acids 40-92 of IGFBP-6 or a fragment thereof

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consisting at least of the 9th to 12th cysteine of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, or IGFBP-5 or at least of the 7th to 10th cysteine of IGFBP-6, based on the atomic coordinates of a crystal consisting of IGF – I and said IGFBP;

- 5 (b) employing said three-dimensional structure and modeling methods to identify a non-proteinaceous compound forming a complex with said IGFBP by hydrophobic binding with amino acids 49, 50, 70, 71 and 74 in the case of IGFBP-5 and in the case of IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4 and IGFBP-6 with the corresponding amino acids according
- 10 to Table 7;
- (c) producing said compound;
- (d) determining the binding between the compound and said IGFBP.

Fig. 1A

10 20 30 40 50 60
 GPETLCGAELVDALQFVCGDRGFYFNKPTGYGSSRRRAPQTGIVDECCFRSCDLRRLEMYCAPLKPAKSA IGF-I
 AYRPSETLCGGELVDLTQFVCGDRGFYFSRPAS--RVSRRSR--GIVEECCFRSCDLALLETYCAT--PAKSE IGF-II
 10 20 30 40 50 60

Fig. 1B

```

P08833   BP1   --QVGVTAG---APWQCAPCSAEKLALCPP----VSASCS-----EVTRSAGCG
P18065   BP2   ---GGGARA--EVLFRCPPTCTPERLAACGPPPVAPPAVAAVAGGARMPCAELVREPGCG
P17936   BP3   ---AGASSGGLGPVVRCEPCDARALAQCAPP----PAVCA-----ELVREPGCG
P22692   BP4   LLAAGPGPSLGDIAIHCPPCSEKLRARP----PVGCE-----ELVREPGCG
P24593   BP5   --YAGPAQS-LGSFVHCEPCDEKALSMCPPS----PLGC-----ELVKEPGCG
P24592   BP6   ---LAASPG--GALARCPGCGQGVQAGC-----PGGCVVE-----EDGGSPAEG
          .       .       .       .       .       .       .       .       .       .
consensus/100%  ...huss.u.....hpC.sCs.th.u.C.....shss.....E.stpsupG

```

[illegible]

```

P08833   BP1   SAPHAAEAGSP-ESPES--TEIT
P18065   BP2   ASPEQVAD-NGDDHSEGGIVENH
P17936   BP3   LLPAPPAPGNASEEEDRSAGSV
P22692   BP4   EIEAIQESLQPSDKDEGDHPNNS
P24593   BP5   REQVKIER-DSREHEEPTTSEMA
P24592   BP6   -APAVAAE-NPKESKQP--AGTA
consensus/100%   .....t...ps.-p.....st..

```

Fig. 2

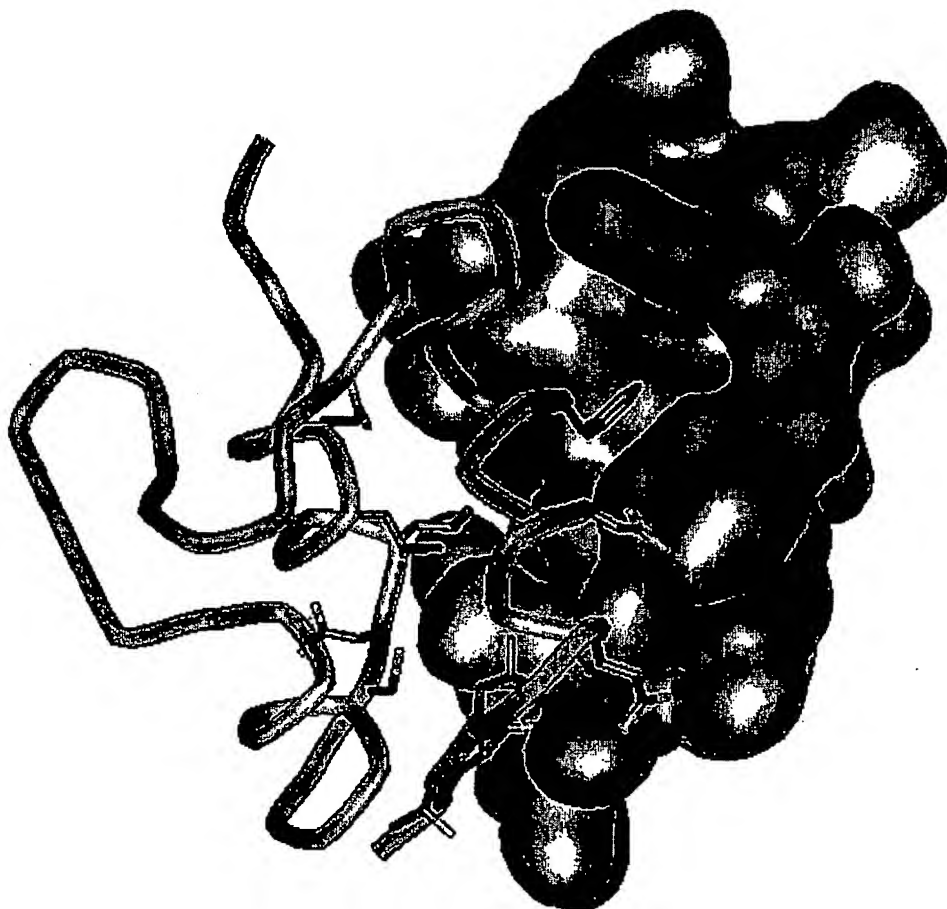


Fig. 3

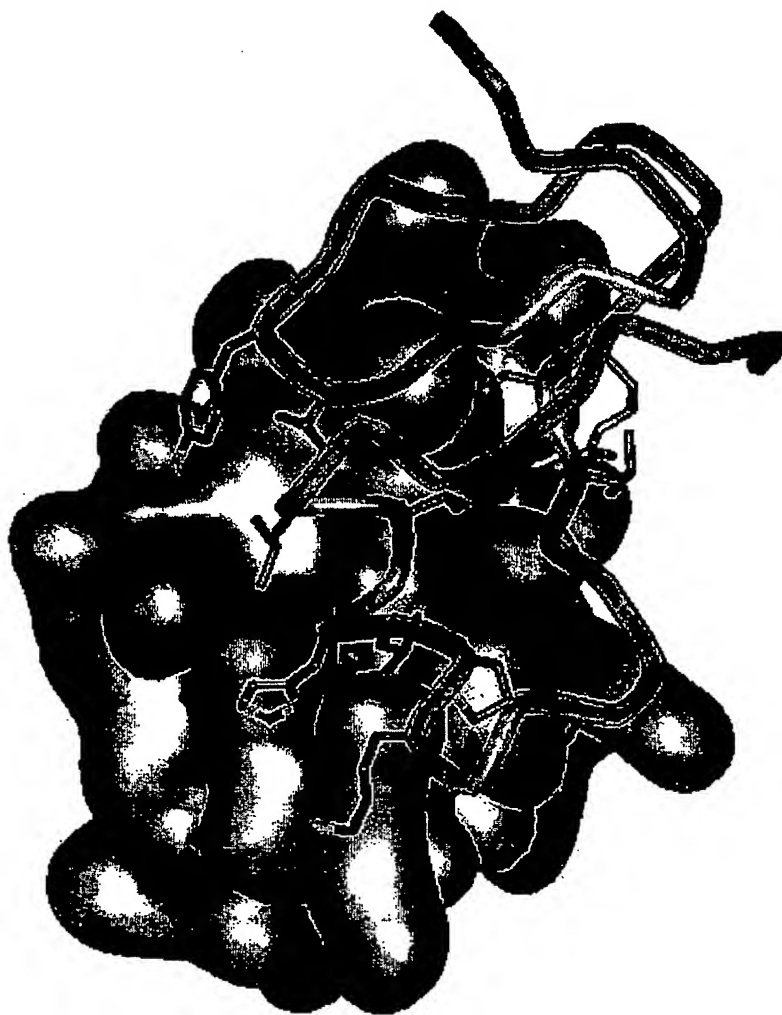


Fig. 4A

	IGF-BP5 Residues										
	Gly48	Val49	Tyr50	Cys60	Glu67	Lys68	Pro69	Leu70	His71	Leu73	Leu74
IGF-Glu3					5	2.9	3.2	3.1	2.5		
IGF-Thr4									3.3		
IGF-Leu5								4.0	4.1		3.7
IGF-Glu9			4.9						4.5		3.9
IGF-Asp12		4.0	2.6								4.8
IGF-Ala13		4.8									4.8
IGF-Ph 16	3.5	3.7		3.7						4.6	

Fig. 4B

	IGF-BP5 residues				
	Tyr50	Lys68	Pro69	Leu70	His71
IGF-Glu3		SC-SC CHB	SC-SC CHB	SC-MC CHB	SB, SC-MC HB
IGF-Thr4					MC-SC CHB
IGF-Asp12	SC-SC HB				

Fig. 5

ATOM	1	CB	PRO	A	2	10.704	22.686	21.862	1.00	48.41	A
ATOM	2	CG	PRO	A	2	11.649	23.436	20.928	1.00	49.62	A
ATOM	3	C	PRO	A	2	9.908	20.943	20.252	1.00	44.31	A
ATOM	4	O	PRO	A	2	9.908	21.594	19.208	1.00	46.80	A
ATOM	5	N	PRO	A	2	12.286	21.170	20.867	1.00	48.45	A
ATOM	6	CD	PRO	A	2	12.881	22.517	20.805	1.00	49.31	A
ATOM	7	CA	PRO	A	2	10.895	21.262	21.370	1.00	47.18	A
ATOM	8	N	GLU	A	3	9.065	19.942	20.475	1.00	40.09	A
ATOM	9	CA	GLU	A	3	8.078	19.540	19.478	1.00	35.47	A
ATOM	10	CB	GLU	A	3	7.425	18.219	19.904	1.00	31.82	A
ATOM	11	CG	GLU	A	3	6.324	17.741	18.977	1.00	30.50	A
ATOM	12	CD	GLU	A	3	5.811	16.350	19.321	1.00	29.25	A
ATOM	13	OE1	GLU	A	3	5.562	16.074	20.512	1.00	29.70	A
ATOM	14	OE2	GLU	A	3	5.640	15.537	18.391	1.00	28.06	A
ATOM	15	C	GLU	A	3	7.002	20.607	19.258	1.00	33.04	A
ATOM	16	O	GLU	A	3	6.553	21.252	20.198	1.00	33.26	A
ATOM	17	N	THR	A	4	6.605	20.796	18.006	1.00	33.52	A
ATOM	18	CA	THR	A	4	5.564	21.761	17.671	1.00	32.64	A
ATOM	19	CB	THR	A	4	6.133	23.010	16.949	1.00	33.01	A
ATOM	20	OG1	THR	A	4	6.850	22.600	15.781	1.00	34.92	A
ATOM	21	CG2	THR	A	4	7.063	23.795	17.868	1.00	33.39	A
ATOM	22	C	THR	A	4	4.539	21.099	16.756	1.00	32.21	A
ATOM	23	O	THR	A	4	4.854	20.143	16.045	1.00	31.41	A
ATOM	24	N	LEU	A	5	3.311	21.607	16.794	1.00	28.70	A
ATOM	25	CA	LEU	A	5	2.225	21.094	15.973	1.00	27.95	A
ATOM	26	CB	LEU	A	5	1.248	20.263	16.811	1.00	30.16	A
ATOM	27	CG	LEU	A	5	1.261	18.739	16.718	1.00	32.01	A
ATOM	28	CD1	LEU	A	5	-0.021	18.204	17.364	1.00	27.14	A
ATOM	29	CD2	LEU	A	5	1.346	18.295	15.262	1.00	28.94	A
ATOM	30	C	LEU	A	5	1.479	22.281	15.380	1.00	27.71	A
ATOM	31	O	LEU	A	5	1.089	23.196	16.102	1.00	25.30	A
ATOM	32	N	CYS	A	6	1.274	22.257	14.068	1.00	27.68	A
ATOM	33	CA	CYS	A	6	0.586	23.344	13.387	1.00	27.97	A
ATOM	34	C	CYS	A	6	-0.467	22.815	12.429	1.00	27.16	A
ATOM	35	O	CYS	A	6	-0.555	21.617	12.189	1.00	25.39	A
ATOM	36	CB	CYS	A	6	1.580	24.177	12.571	1.00	31.40	A
ATOM	37	SG	CYS	A	6	3.038	24.809	13.457	1.00	38.95	A
ATOM	38	N	GLY	A	7	-1.255	23.736	11.887	1.00	26.22	A
ATOM	39	CA	GLY	A	7	-2.279	23.399	10.914	1.00	25.51	A
ATOM	40	C	GLY	A	7	-3.307	22.330	11.229	1.00	25.84	A
ATOM	41	O	GLY	A	7	-3.814	22.213	12.351	1.00	24.26	A
ATOM	42	N	ALA	A	8	-3.633	21.559	10.199	1.00	22.75	A
ATOM	43	CA	ALA	A	8	-4.622	20.495	10.301	1.00	23.80	A
ATOM	44	CB	ALA	A	8	-4.783	19.804	8.946	1.00	23.28	A
ATOM	45	C	ALA	A	8	-4.242	19.484	11.371	1.00	22.59	A
ATOM	46	O	ALA	A	8	-5.102	18.969	12.073	1.00	23.77	A
ATOM	47	N	GLU	A	9	-2.950	19.205	11.511	1.00	23.09	A
ATOM	48	CA	GLU	A	9	-2.528	18.244	12.522	1.00	24.67	A
ATOM	49	CB	GLU	A	9	-1.047	17.911	12.370	1.00	26.80	A
ATOM	50	CG	GLU	A	9	-0.741	17.101	11.132	1.00	32.24	A
ATOM	51	CD	GLU	A	9	0.698	16.651	11.096	1.00	36.38	A
ATOM	52	OE1	GLU	A	9	1.588	17.526	11.148	1.00	39.56	A
ATOM	53	OE2	GLU	A	9	0.936	15.427	11.022	1.00	37.49	A
ATOM	54	C	GLU	A	9	-2.799	18.730	13.935	1.00	21.39	A
ATOM	55	O	GLU	A	9	-3.115	17.934	14.812	1.00	21.62	A
ATOM	56	N	LEU	A	10	-2.660	20.032	14.163	1.00	20.36	A
ATOM	57	CA	LEU	A	10	-2.915	20.588	15.484	1.00	19.60	A

Fig. 5 (contd.)

ATOM	58	CB	LEU	A	10	-2.488	22.061	15.530	1.00	20.83	A
ATOM	59	CG	LEU	A	10	-2.900	22.790	16.813	1.00	23.06	A
ATOM	60	CD1	LEU	A	10	-2.279	22.087	18.012	1.00	22.52	A
ATOM	61	CD2	LEU	A	10	-2.466	24.252	16.753	1.00	24.50	A
ATOM	62	C	LEU	A	10	-4.411	20.461	15.818	1.00	18.65	A
ATOM	63	O	LEU	A	10	-4.793	20.141	16.945	1.00	16.88	A
ATOM	64	N	VAL	A	11	-5.253	20.732	14.830	1.00	15.88	A
ATOM	65	CA	VAL	A	11	-6.695	20.633	15.013	1.00	18.59	A
ATOM	66	CB	VAL	A	11	-7.455	21.193	13.786	1.00	21.42	A
ATOM	67	CG1	VAL	A	11	-8.955	20.957	13.935	1.00	21.23	A
ATOM	68	CG2	VAL	A	11	-7.185	22.691	13.659	1.00	23.65	A
ATOM	69	C	VAL	A	11	-7.097	19.177	15.250	1.00	18.86	A
ATOM	70	O	VAL	A	11	-7.914	18.891	16.116	1.00	19.03	A
ATOM	71	N	ASP	A	12	-6.520	18.253	14.489	1.00	19.12	A
ATOM	72	CA	ASP	A	12	-6.853	16.845	14.679	1.00	17.94	A
ATOM	73	CB	ASP	A	12	-6.173	15.977	13.615	1.00	18.04	A
ATOM	74	CG	ASP	A	12	-6.792	16.155	12.239	1.00	18.18	A
ATOM	75	OD1	ASP	A	12	-7.888	16.747	12.148	1.00	17.41	A
ATOM	76	OD2	ASP	A	12	-6.190	15.696	11.250	1.00	17.12	A
ATOM	77	C	ASP	A	12	-6.449	16.374	16.074	1.00	17.94	A
ATOM	78	O	ASP	A	12	-7.192	15.636	16.715	1.00	17.22	A
ATOM	79	N	ALA	A	13	-5.277	16.804	16.538	1.00	17.64	A
ATOM	80	CA	ALA	A	13	-4.796	16.430	17.870	1.00	19.37	A
ATOM	81	CB	ALA	A	13	-3.371	16.986	18.101	1.00	13.96	A
ATOM	82	C	ALA	A	13	-5.751	16.943	18.952	1.00	19.44	A
ATOM	83	O	ALA	A	13	-6.028	16.242	19.925	1.00	22.14	A
ATOM	84	N	LEU	A	14	-6.245	18.168	18.783	1.00	21.01	A
ATOM	85	CA	LEU	A	14	-7.188	18.760	19.735	1.00	22.46	A
ATOM	86	CB	LEU	A	14	-7.482	20.216	19.359	1.00	22.01	A
ATOM	87	CG	LEU	A	14	-6.434	21.234	19.809	1.00	24.59	A
ATOM	88	CD1	LEU	A	14	-6.611	22.540	19.059	1.00	26.66	A
ATOM	89	CD2	LEU	A	14	-6.554	21.439	21.309	1.00	24.62	A
ATOM	90	C	LEU	A	14	-8.498	17.976	19.777	1.00	21.46	A
ATOM	91	O	LEU	A	14	-9.054	17.715	20.848	1.00	22.17	A
ATOM	92	N	GLN	A	15	-8.993	17.615	18.600	1.00	21.30	A
ATOM	93	CA	GLN	A	15	-10.224	16.845	18.487	1.00	22.52	A
ATOM	94	CB	GLN	A	15	-10.577	16.631	17.010	1.00	21.07	A
ATOM	95	CG	GLN	A	15	-11.745	15.682	16.800	1.00	27.80	A
ATOM	96	CD	GLN	A	15	-13.071	16.277	17.247	1.00	29.64	A
ATOM	97	OE1	GLN	A	15	-13.943	15.568	17.747	1.00	35.24	A
ATOM	98	NE2	GLN	A	15	-13.231	17.579	17.057	1.00	28.27	A
ATOM	99	C	GLN	A	15	-10.040	15.491	19.174	1.00	22.02	A
ATOM	100	O	GLN	A	15	-10.938	15.003	19.860	1.00	22.22	A
ATOM	101	N	PHE	A	16	-8.866	14.893	18.997	1.00	21.16	A
ATOM	102	CA	PHE	A	16	-8.584	13.596	19.606	1.00	24.00	A
ATOM	103	CB	PHE	A	16	-7.271	13.019	19.056	1.00	23.82	A
ATOM	104	CG	PHE	A	16	-6.952	11.640	19.578	1.00	29.07	A
ATOM	105	CD1	PHE	A	16	-7.795	10.566	19.303	1.00	31.09	A
ATOM	106	CD2	PHE	A	16	-5.821	11.419	20.357	1.00	31.17	A
ATOM	107	CE1	PHE	A	16	-7.518	9.289	19.796	1.00	32.48	A
ATOM	108	CE2	PHE	A	16	-5.532	10.143	20.857	1.00	34.53	A
ATOM	109	CZ	PHE	A	16	-6.382	9.078	20.576	1.00	32.86	A
ATOM	110	C	PHE	A	16	-8.499	13.703	21.133	1.00	24.10	A
ATOM	111	O	PHE	A	16	-9.121	12.927	21.854	1.00	23.16	A
ATOM	112	N	VAL	A	17	-7.741	14.677	21.623	1.00	22.26	A
ATOM	113	CA	VAL	A	17	-7.578	14.856	23.062	1.00	24.25	A
ATOM	114	CB	VAL	A	17	-6.457	15.886	23.360	1.00	25.26	A
ATOM	115	CG1	VAL	A	17	-6.397	16.202	24.848	1.00	24.79	A

Fig. 5 (contd.)

ATOM	116	CG2	VAL	A	17	-5.126	15.336	22.893	1.00	22.56	A
ATOM	117	C	VAL	A	17	-8.859	15.289	23.775	1.00	25.86	A
ATOM	118	O	VAL	A	17	-9.221	14.726	24.803	1.00	26.11	A
ATOM	119	N	CYS	A	18	-9.551	16.275	23.215	1.00	26.25	A
ATOM	120	CA	CYS	A	18	-10.772	16.806	23.819	1.00	27.65	A
ATOM	121	C	CYS	A	18	-12.067	16.036	23.553	1.00	29.15	A
ATOM	122	O	CYS	A	18	-12.993	16.092	24.358	1.00	31.90	A
ATOM	123	CB	CYS	A	18	-10.949	18.263	23.392	1.00	22.29	A
ATOM	124	SG	CYS	A	18	-9.517	19.306	23.795	1.00	21.36	A
ATOM	125	N	GLY	A	19	-12.144	15.337	22.426	1.00	31.46	A
ATOM	126	CA	GLY	A	19	-13.340	14.568	22.121	1.00	31.99	A
ATOM	127	C	GLY	A	19	-14.626	15.372	22.043	1.00	34.26	A
ATOM	128	O	GLY	A	19	-14.649	16.479	21.504	1.00	35.27	A
ATOM	129	N	ASP	A	20	-15.700	14.816	22.594	1.00	34.61	A
ATOM	130	CA	ASP	A	20	-17.003	15.470	22.575	1.00	37.53	A
ATOM	131	CB	ASP	A	20	-18.094	14.486	23.016	1.00	40.28	A
ATOM	132	CG	ASP	A	20	-17.768	13.810	24.322	1.00	44.00	A
ATOM	133	OD1	ASP	A	20	-17.483	14.523	25.305	1.00	47.54	A
ATOM	134	OD2	ASP	A	20	-17.797	12.563	24.369	1.00	50.02	A
ATOM	135	C	ASP	A	20	-17.090	16.743	23.416	1.00	35.72	A
ATOM	136	O	ASP	A	20	-18.071	17.475	23.329	1.00	37.26	A
ATOM	137	N	ARG	A	21	-16.073	17.016	24.225	1.00	35.25	A
ATOM	138	CA	ARG	A	21	-16.083	18.228	25.041	1.00	32.99	A
ATOM	139	CB	ARG	A	21	-14.920	18.218	26.028	1.00	33.07	A
ATOM	140	CG	ARG	A	21	-14.955	17.084	27.027	1.00	36.04	A
ATOM	141	CD	ARG	A	21	-13.650	17.027	27.788	1.00	38.67	A
ATOM	142	NE	ARG	A	21	-13.361	18.293	28.449	1.00	40.34	A
ATOM	143	CZ	ARG	A	21	-12.237	18.547	29.109	1.00	41.42	A
ATOM	144	NH1	ARG	A	21	-11.296	17.618	29.194	1.00	39.64	A
ATOM	145	NH2	ARG	A	21	-12.057	19.728	29.688	1.00	42.26	A
ATOM	146	C	ARG	A	21	-15.962	19.473	24.166	1.00	31.94	A
ATOM	147	O	ARG	A	21	-16.540	20.513	24.465	1.00	32.47	A
ATOM	148	N	GLY	A	22	-15.214	19.365	23.075	1.00	29.01	A
ATOM	149	CA	GLY	A	22	-15.024	20.521	22.221	1.00	26.06	A
ATOM	150	C	GLY	A	22	-13.815	21.261	22.757	1.00	24.26	A
ATOM	151	O	GLY	A	22	-13.289	20.890	23.802	1.00	22.46	A
ATOM	152	N	PHE	A	23	-13.373	22.306	22.066	1.00	22.86	A
ATOM	153	CA	PHE	A	23	-12.198	23.045	22.512	1.00	22.17	A
ATOM	154	CB	PHE	A	23	-10.933	22.312	22.039	1.00	20.23	A
ATOM	155	CG	PHE	A	23	-10.925	22.004	20.560	1.00	21.99	A
ATOM	156	CD1	PHE	A	23	-10.457	22.938	19.642	1.00	22.24	A
ATOM	157	CD2	PHE	A	23	-11.414	20.787	20.086	1.00	23.68	A
ATOM	158	CE1	PHE	A	23	-10.474	22.670	18.270	1.00	25.86	A
ATOM	159	CE2	PHE	A	23	-11.439	20.506	18.713	1.00	25.11	A
ATOM	160	CZ	PHE	A	23	-10.968	21.449	17.805	1.00	25.09	A
ATOM	161	C	PHE	A	23	-12.181	24.489	22.024	1.00	21.88	A
ATOM	162	O	PHE	A	23	-12.973	24.877	21.162	1.00	23.17	A
ATOM	163	N	TYR	A	24	-11.274	25.279	22.587	1.00	21.43	A
ATOM	164	CA	TYR	A	24	-11.134	26.683	22.215	1.00	22.21	A
ATOM	165	CB	TYR	A	24	-11.273	27.587	23.439	1.00	22.91	A
ATOM	166	CG	TYR	A	24	-12.633	27.540	24.077	1.00	23.87	A
ATOM	167	CD1	TYR	A	24	-13.779	27.855	23.345	1.00	23.25	A
ATOM	168	CE1	TYR	A	24	-15.036	27.804	23.928	1.00	23.74	A
ATOM	169	CD2	TYR	A	24	-12.781	27.175	25.411	1.00	25.69	A
ATOM	170	CE2	TYR	A	24	-14.034	27.125	26.003	1.00	25.28	A
ATOM	171	CZ	TYR	A	24	-15.153	27.437	25.260	1.00	26.80	A
ATOM	172	OH	TYR	A	24	-16.389	27.374	25.853	1.00	30.71	A
ATOM	173	C	TYR	A	24	-9.784	26.938	21.576	1.00	23.50	A

Fig. 5 (contd.)

ATOM	174	O	TYR	A	24	-8.853	26.141	21.727	1.00	23.43	A
ATOM	175	N	PHE	A	25	-9.682	28.064	20.875	1.00	22.72	A
ATOM	176	CA	PHE	A	25	-8.448	28.451	20.208	1.00	25.06	A
ATOM	177	CB	PHE	A	25	-8.706	28.776	18.731	1.00	24.67	A
ATOM	178	CG	PHE	A	25	-8.965	27.582	17.878	1.00	27.79	A
ATOM	179	CD1	PHE	A	25	-10.263	27.237	17.517	1.00	27.21	A
ATOM	180	CD2	PHE	A	25	-7.909	26.803	17.417	1.00	28.61	A
ATOM	181	CE1	PHE	A	25	-10.506	26.131	16.705	1.00	30.80	A
ATOM	182	CE2	PHE	A	25	-8.143	25.692	16.604	1.00	30.69	A
ATOM	183	CZ	PHE	A	25	-9.445	25.358	16.248	1.00	28.62	A
ATOM	184	C	PHE	A	25	-7.732	29.657	20.804	1.00	26.11	A
ATOM	185	O	PHE	A	25	-6.514	29.635	20.992	1.00	26.19	A
ATOM	186	N	ASN	A	26	-8.482	30.714	21.095	1.00	27.47	A
ATOM	187	CA	ASN	A	26	-7.865	31.948	21.574	1.00	29.42	A
ATOM	188	CB	ASN	A	26	-8.809	33.129	21.342	1.00	28.22	A
ATOM	189	CG	ASN	A	26	-8.057	34.427	21.126	1.00	31.48	A
ATOM	190	OD1	ASN	A	26	-7.194	34.509	20.254	1.00	34.77	A
ATOM	191	ND2	ASN	A	26	-8.372	35.441	21.914	1.00	31.59	A
ATOM	192	C	ASN	A	26	-7.345	31.989	23.000	1.00	29.47	A
ATOM	193	O	ASN	A	26	-6.367	32.680	23.280	1.00	29.14	A
ATOM	194	N	LYS	A	27	-7.995	31.263	23.901	1.00	29.57	A
ATOM	195	CA	LYS	A	27	-7.569	31.244	25.293	1.00	30.66	A
ATOM	196	CB	LYS	A	27	-8.362	32.264	26.118	1.00	30.77	A
ATOM	197	CG	LYS	A	27	-8.294	33.694	25.627	1.00	34.21	A
ATOM	198	CD	LYS	A	27	-6.919	34.297	25.856	1.00	37.48	A
ATOM	199	CE	LYS	A	27	-6.920	35.784	25.530	1.00	40.53	A
ATOM	200	NZ	LYS	A	27	-5.582	36.399	25.766	1.00	44.14	A
ATOM	201	C	LYS	A	27	-7.821	29.864	25.867	1.00	31.08	A
ATOM	202	O	LYS	A	27	-8.707	29.148	25.403	1.00	30.45	A
ATOM	203	N	PRO	A	28	-7.032	29.464	26.875	1.00	31.82	A
ATOM	204	CD	PRO	A	28	-5.841	30.142	27.421	1.00	32.67	A
ATOM	205	CA	PRO	A	28	-7.215	28.152	27.496	1.00	33.47	A
ATOM	206	CB	PRO	A	28	-5.895	27.932	28.225	1.00	34.86	A
ATOM	207	CG	PRO	A	28	-5.534	29.319	28.659	1.00	34.31	A
ATOM	208	C	PRO	A	28	-8.391	28.272	28.459	1.00	35.58	A
ATOM	209	O	PRO	A	28	-8.724	29.377	28.900	1.00	32.43	A
ATOM	210	N	THR	A	29	-9.025	27.150	28.774	1.00	37.76	A
ATOM	211	CA	THR	A	29	-10.154	27.165	29.695	1.00	41.89	A
ATOM	212	CB	THR	A	29	-10.940	25.836	29.640	1.00	41.67	A
ATOM	213	OG1	THR	A	29	-11.557	25.697	28.353	1.00	43.11	A
ATOM	214	CG2	THR	A	29	-12.019	25.805	30.717	1.00	42.37	A
ATOM	215	C	THR	A	29	-9.631	27.378	31.110	1.00	45.51	A
ATOM	216	O	THR	A	29	-8.550	26.903	31.456	1.00	44.18	A
ATOM	217	N	GLY	A	30	-10.390	28.112	31.916	1.00	50.36	A
ATOM	218	CA	GLY	A	30	-9.985	28.354	33.288	1.00	56.95	A
ATOM	219	C	GLY	A	30	-8.974	29.468	33.469	1.00	61.71	A
ATOM	220	O	GLY	A	30	-9.261	30.628	33.172	1.00	63.19	A
ATOM	221	N	TYR	A	31	-7.787	29.107	33.955	1.00	65.17	A
ATOM	222	CA	TYR	A	31	-6.715	30.067	34.209	1.00	68.15	A
ATOM	223	CB	TYR	A	31	-6.541	31.020	33.015	1.00	70.95	A
ATOM	224	CG	TYR	A	31	-5.399	32.012	33.154	1.00	73.49	A
ATOM	225	CD1	TYR	A	31	-5.486	33.094	34.033	1.00	74.34	A
ATOM	226	CE1	TYR	A	31	-4.445	34.004	34.167	1.00	76.14	A
ATOM	227	CD2	TYR	A	31	-4.229	31.868	32.406	1.00	74.79	A
ATOM	228	CE2	TYR	A	31	-3.177	32.778	32.533	1.00	76.14	A
ATOM	229	CZ	TYR	A	31	-3.294	33.842	33.416	1.00	76.89	A
ATOM	230	OH	TYR	A	31	-2.264	34.743	33.556	1.00	77.25	A
ATOM	231	C	TYR	A	31	-7.058	30.842	35.479	1.00	68.59	A

Fig. 5 (contd.)

ATOM	232	OT1	TYR	A	31	-7.492	32.010	35.373	1.00	69.00	A
ATOM	233	OXT	TYR	A	31	-6.913	30.253	36.573	1.00	68.68	A
ATOM	575	CB	THR	C	41	-0.918	33.149	24.393	1.00	42.36	C
ATOM	576	OG1	THR	C	41	-0.923	33.851	23.141	1.00	46.28	C
ATOM	577	CG2	THR	C	41	-0.325	31.766	24.185	1.00	44.57	C
ATOM	578	C	THR	C	41	-3.105	31.956	24.138	1.00	36.92	C
ATOM	579	O	THR	C	41	-3.705	31.060	24.730	1.00	36.68	C
ATOM	580	N	THR	C	41	-2.348	32.688	26.380	1.00	41.52	C
ATOM	581	CA	THR	C	41	-2.354	33.032	24.928	1.00	40.17	C
ATOM	582	N	GLY	C	42	-3.064	32.049	22.809	1.00	33.32	C
ATOM	583	CA	GLY	C	42	-3.763	31.092	21.963	1.00	30.89	C
ATOM	584	C	GLY	C	42	-2.997	29.810	21.687	1.00	29.22	C
ATOM	585	O	GLY	C	42	-1.768	29.789	21.730	1.00	27.67	C
ATOM	586	N	ILE	C	43	-3.726	28.738	21.383	1.00	27.59	C
ATOM	587	CA	ILE	C	43	-3.106	27.441	21.115	1.00	26.54	C
ATOM	588	CB	ILE	C	43	-4.184	26.327	20.976	1.00	26.50	C
ATOM	589	CG2	ILE	C	43	-5.039	26.562	19.734	1.00	25.25	C
ATOM	590	CG1	ILE	C	43	-3.513	24.950	20.910	1.00	28.05	C
ATOM	591	CD	ILE	C	43	-2.830	24.535	22.192	1.00	29.33	C
ATOM	592	C	ILE	C	43	-2.186	27.427	19.878	1.00	26.00	C
ATOM	593	O	ILE	C	43	-1.163	26.742	19.875	1.00	23.28	C
ATOM	594	N	VAL	C	44	-2.544	28.166	18.831	1.00	27.62	C
ATOM	595	CA	VAL	C	44	-1.704	28.221	17.628	1.00	31.20	C
ATOM	596	CB	VAL	C	44	-2.353	29.056	16.502	1.00	32.97	C
ATOM	597	CG1	VAL	C	44	-1.364	29.237	15.353	1.00	33.20	C
ATOM	598	CG2	VAL	C	44	-3.614	28.368	16.001	1.00	33.05	C
ATOM	599	C	VAL	C	44	-0.354	28.848	17.968	1.00	33.88	C
ATOM	600	O	VAL	C	44	0.694	28.350	17.550	1.00	33.44	C
ATOM	601	N	ASP	C	45	-0.383	29.944	18.724	1.00	35.62	C
ATOM	602	CA	ASP	C	45	0.844	30.623	19.132	1.00	36.95	C
ATOM	603	CB	ASP	C	45	0.524	31.847	19.996	1.00	38.58	C
ATOM	604	CG	ASP	C	45	0.486	33.135	19.198	1.00	39.81	C
ATOM	605	OD1	ASP	C	45	-0.356	34.000	19.516	1.00	42.11	C
ATOM	606	OD2	ASP	C	45	1.300	33.291	18.265	1.00	38.39	C
ATOM	607	C	ASP	C	45	1.719	29.668	19.931	1.00	37.42	C
ATOM	608	O	ASP	C	45	2.888	29.467	19.608	1.00	40.61	C
ATOM	609	N	GLU	C	46	1.150	29.082	20.980	1.00	35.14	C
ATOM	610	CA	GLU	C	46	1.903	28.154	21.810	1.00	34.98	C
ATOM	611	CB	GLU	C	46	1.062	27.683	22.999	1.00	35.29	C
ATOM	612	CG	GLU	C	46	0.816	28.736	24.070	1.00	41.36	C
ATOM	613	CD	GLU	C	46	2.103	29.232	24.714	1.00	44.18	C
ATOM	614	OE1	GLU	C	46	2.938	28.388	25.107	1.00	44.06	C
ATOM	615	OE2	GLU	C	46	2.277	30.463	24.833	1.00	44.52	C
ATOM	616	C	GLU	C	46	2.383	26.937	21.027	1.00	33.34	C
ATOM	617	O	GLU	C	46	3.577	26.795	20.765	1.00	33.89	C
ATOM	618	N	CYS	C	47	1.444	26.077	20.637	1.00	29.37	C
ATOM	619	CA	CYS	C	47	1.769	24.844	19.923	1.00	29.96	C
ATOM	620	C	CYS	C	47	2.496	24.937	18.595	1.00	29.21	C
ATOM	621	O	CYS	C	47	3.383	24.132	18.320	1.00	30.38	C
ATOM	622	CB	CYS	C	47	0.504	23.986	19.756	1.00	26.58	C
ATOM	623	SG	CYS	C	47	0.250	22.978	21.239	1.00	26.80	C
ATOM	624	N	CYS	C	48	2.153	25.919	17.777	1.00	28.58	C
ATOM	625	CA	CYS	C	48	2.792	26.029	16.479	1.00	30.29	C
ATOM	626	C	CYS	C	48	4.138	26.760	16.422	1.00	32.48	C
ATOM	627	O	CYS	C	48	5.013	26.394	15.629	1.00	31.64	C
ATOM	628	CB	CYS	C	48	1.825	26.666	15.482	1.00	32.91	C
ATOM	629	SG	CYS	C	48	2.559	26.749	13.832	1.00	41.22	C
ATOM	630	N	PHE	C	49	4.318	27.775	17.261	1.00	32.39	C

Fig. 5 (contd.)

ATOM	631	CA	PHE	C	49	5.559	28.545	17.247	1.00	33.94	C
ATOM	632	CB	PHE	C	49	5.232	30.028	17.057	1.00	32.89	C
ATOM	633	CG	PHE	C	49	4.520	30.322	15.771	1.00	30.98	C
ATOM	634	CD1	PHE	C	49	5.168	30.155	14.552	1.00	30.72	C
ATOM	635	CD2	PHE	C	49	3.194	30.737	15.774	1.00	29.66	C
ATOM	636	CE1	PHE	C	49	4.504	30.397	13.353	1.00	31.20	C
ATOM	637	CE2	PHE	C	49	2.523	30.981	14.584	1.00	29.98	C
ATOM	638	CZ	PHE	C	49	3.178	30.810	13.371	1.00	31.19	C
ATOM	639	C	PHE	C	49	6.477	28.382	18.452	1.00	34.62	C
ATOM	640	O	PHE	C	49	7.656	28.724	18.376	1.00	36.79	C
ATOM	641	N	ARG	C	50	5.956	27.859	19.558	1.00	35.31	C
ATOM	642	CA	ARG	C	50	6.765	27.687	20.759	1.00	37.16	C
ATOM	643	CB	ARG	C	50	6.127	28.456	21.922	1.00	39.61	C
ATOM	644	CG	ARG	C	50	6.136	29.980	21.735	1.00	45.48	C
ATOM	645	CD	ARG	C	50	5.256	30.688	22.769	1.00	49.13	C
ATOM	646	NE	ARG	C	50	5.220	32.139	22.566	1.00	54.47	C
ATOM	647	CZ	ARG	C	50	4.361	32.962	23.171	1.00	57.80	C
ATOM	648	NH1	ARG	C	50	3.455	32.485	24.018	1.00	58.23	C
ATOM	649	NH2	ARG	C	50	4.410	34.271	22.943	1.00	58.43	C
ATOM	650	C	ARG	C	50	6.980	26.223	21.149	1.00	37.35	C
ATOM	651	O	ARG	C	50	8.011	25.632	20.832	1.00	37.72	C
ATOM	652	N	SER	C	51	6.009	25.636	21.836	1.00	36.82	C
ATOM	653	CA	SER	C	51	6.123	24.245	22.259	1.00	36.64	C
ATOM	654	CB	SER	C	51	6.913	24.160	23.569	1.00	36.90	C
ATOM	655	OG	SER	C	51	6.843	22.858	24.116	1.00	42.09	C
ATOM	656	C	SER	C	51	4.749	23.614	22.450	1.00	34.49	C
ATOM	657	O	SER	C	51	3.830	24.255	22.959	1.00	35.64	C
ATOM	658	N	CYS	C	52	4.620	22.353	22.047	1.00	31.83	C
ATOM	659	CA	CYS	C	52	3.355	21.636	22.171	1.00	27.84	C
ATOM	660	C	CYS	C	52	3.572	20.302	22.862	1.00	27.84	C
ATOM	661	O	CYS	C	52	4.584	19.641	22.645	1.00	29.73	C
ATOM	662	CB	CYS	C	52	2.765	21.374	20.786	1.00	26.29	C
ATOM	663	SG	CYS	C	52	0.950	21.153	20.677	1.00	25.58	C
ATOM	664	N	ASP	C	53	2.622	19.926	23.706	1.00	28.33	C
ATOM	665	CA	ASP	C	53	2.649	18.649	24.398	1.00	30.31	C
ATOM	666	CB	ASP	C	53	3.561	18.679	25.634	1.00	33.16	C
ATOM	667	CG	ASP	C	53	3.229	19.806	26.596	1.00	35.20	C
ATOM	668	OD1	ASP	C	53	2.036	20.048	26.866	1.00	34.50	C
ATOM	669	OD2	ASP	C	53	4.179	20.440	27.100	1.00	39.85	C
ATOM	670	C	ASP	C	53	1.216	18.346	24.795	1.00	30.38	C
ATOM	671	O	ASP	C	53	0.338	19.207	24.664	1.00	29.48	C
ATOM	672	N	LEU	C	54	0.974	17.129	25.266	1.00	29.20	C
ATOM	673	CA	LEU	C	54	-0.368	16.725	25.658	1.00	31.11	C
ATOM	674	CB	LEU	C	54	-0.374	15.261	26.110	1.00	31.37	C
ATOM	675	CG	LEU	C	54	-0.354	14.214	24.991	1.00	34.52	C
ATOM	676	CD1	LEU	C	54	-0.302	12.822	25.591	1.00	36.09	C
ATOM	677	CD2	LEU	C	54	-1.603	14.361	24.123	1.00	35.95	C
ATOM	678	C	LEU	C	54	-1.009	17.589	26.734	1.00	30.74	C
ATOM	679	O	LEU	C	54	-2.207	17.870	26.667	1.00	27.74	C
ATOM	680	N	ARG	C	55	-0.227	18.012	27.725	1.00	31.56	C
ATOM	681	CA	ARG	C	55	-0.782	18.830	28.800	1.00	34.34	C
ATOM	682	CB	ARG	C	55	0.251	19.043	29.911	1.00	38.80	C
ATOM	683	CG	ARG	C	55	-0.257	19.915	31.051	1.00	47.66	C
ATOM	684	CD	ARG	C	55	0.785	20.080	32.153	1.00	55.42	C
ATOM	685	NE	ARG	C	55	0.337	21.016	33.183	1.00	61.23	C
ATOM	686	CZ	ARG	C	55	1.059	21.368	34.243	1.00	65.09	C
ATOM	687	NH1	ARG	C	55	2.273	20.862	34.423	1.00	66.31	C
ATOM	688	NH2	ARG	C	55	0.571	22.237	35.120	1.00	66.42	C

Fig. 5 (contd.)

ATOM	689	C	ARG	C	55	-1.271	20.180	28.282	1.00	32.74	C
ATOM	690	O	ARG	C	55	-2.300	20.682	28.726	1.00	32.45	C
ATOM	691	N	ARG	C	56	-0.535	20.757	27.336	1.00	31.40	C
ATOM	692	CA	ARG	C	56	-0.908	22.041	26.760	1.00	33.43	C
ATOM	693	CB	ARG	C	56	0.174	22.509	25.786	1.00	36.65	C
ATOM	694	CG	ARG	C	56	-0.072	23.894	25.210	1.00	44.36	C
ATOM	695	CD	ARG	C	56	0.139	24.966	26.264	1.00	50.86	C
ATOM	696	NE	ARG	C	56	1.538	25.052	26.675	1.00	53.58	C
ATOM	697	CZ	ARG	C	56	1.985	25.846	27.641	1.00	56.11	C
ATOM	698	NH1	ARG	C	56	1.141	26.627	28.304	1.00	56.49	C
ATOM	699	NH2	ARG	C	56	3.277	25.865	27.942	1.00	57.01	C
ATOM	700	C	ARG	C	56	-2.250	21.930	26.026	1.00	31.70	C
ATOM	701	O	ARG	C	56	-3.121	22.789	26.171	1.00	30.50	C
ATOM	702	N	LEU	C	57	-2.408	20.859	25.250	1.00	30.08	C
ATOM	703	CA	LEU	C	57	-3.631	20.619	24.485	1.00	28.56	C
ATOM	704	CB	LEU	C	57	-3.484	19.337	23.643	1.00	24.71	C
ATOM	705	CG	LEU	C	57	-2.498	19.427	22.467	1.00	25.16	C
ATOM	706	CD1	LEU	C	57	-2.266	18.052	21.855	1.00	25.45	C
ATOM	707	CD2	LEU	C	57	-3.045	20.381	21.417	1.00	25.04	C
ATOM	708	C	LEU	C	57	-4.873	20.511	25.366	1.00	28.56	C
ATOM	709	O	LEU	C	57	-5.903	21.120	25.077	1.00	26.60	C
ATOM	710	N	GLU	C	58	-4.771	19.740	26.444	1.00	29.27	C
ATOM	711	CA	GLU	C	58	-5.899	19.546	27.349	1.00	29.45	C
ATOM	712	CB	GLU	C	58	-5.539	18.517	28.426	1.00	32.63	C
ATOM	713	CG	GLU	C	58	-5.251	17.142	27.858	1.00	41.62	C
ATOM	714	CD	GLU	C	58	-5.007	16.101	28.931	1.00	46.45	C
ATOM	715	OE1	GLU	C	58	-5.923	15.868	29.748	1.00	47.01	C
ATOM	716	OE2	GLU	C	58	-3.901	15.518	28.954	1.00	47.37	C
ATOM	717	C	GLU	C	58	-6.379	20.838	28.003	1.00	26.98	C
ATOM	718	O	GLU	C	58	-7.548	20.960	28.356	1.00	26.79	C
ATOM	719	N	MET	C	59	-5.485	21.805	28.156	1.00	25.43	C
ATOM	720	CA	MET	C	59	-5.857	23.079	28.760	1.00	27.07	C
ATOM	721	CB	MET	C	59	-4.622	23.965	28.932	1.00	28.76	C
ATOM	722	CG	MET	C	59	-3.616	23.475	29.953	1.00	33.93	C
ATOM	723	SD	MET	C	59	-2.160	24.548	29.943	1.00	42.31	C
ATOM	724	CE	MET	C	59	-2.861	26.092	30.568	1.00	40.03	C
ATOM	725	C	MET	C	59	-6.883	23.827	27.907	1.00	26.07	C
ATOM	726	O	MET	C	59	-7.644	24.648	28.421	1.00	26.94	C
ATOM	727	N	TYR	C	60	-6.904	23.546	26.607	1.00	23.71	C
ATOM	728	CA	TYR	C	60	-7.828	24.228	25.707	1.00	24.20	C
ATOM	729	CB	TYR	C	60	-7.141	24.536	24.367	1.00	22.53	C
ATOM	730	CG	TYR	C	60	-6.054	25.587	24.496	1.00	23.27	C
ATOM	731	CD1	TYR	C	60	-4.794	25.257	25.000	1.00	23.79	C
ATOM	732	CE1	TYR	C	60	-3.811	26.225	25.170	1.00	25.75	C
ATOM	733	CD2	TYR	C	60	-6.301	26.923	24.163	1.00	24.44	C
ATOM	734	CE2	TYR	C	60	-5.323	27.903	24.336	1.00	24.69	C
ATOM	735	CZ	TYR	C	60	-4.080	27.545	24.839	1.00	26.37	C
ATOM	736	OH	TYR	C	60	-3.112	28.503	25.020	1.00	29.55	C
ATOM	737	C	TYR	C	60	-9.145	23.507	25.468	1.00	24.59	C
ATOM	738	O	TYR	C	60	-10.004	24.015	24.750	1.00	23.53	C
ATOM	739	N	CYS	C	61	-9.314	22.327	26.064	1.00	25.69	C
ATOM	740	CA	CYS	C	61	-10.568	21.598	25.912	1.00	25.12	C
ATOM	741	C	CYS	C	61	-11.642	22.398	26.645	1.00	28.22	C
ATOM	742	O	CYS	C	61	-11.346	23.095	27.609	1.00	26.64	C
ATOM	743	CB	CYS	C	61	-10.480	20.197	26.538	1.00	23.04	C
ATOM	744	SG	CYS	C	61	-9.281	19.035	25.795	1.00	24.30	C
ATOM	745	N	ALA	C	62	-12.885	22.305	26.188	1.00	31.44	C
ATOM	746	CA	ALA	C	62	-13.976	23.021	26.840	1.00	35.50	C

Fig. 5 (contd.)

ATOM	747	CB	ALA	C	62	-15.165	23.149	25.889	1.00	34.68	C
ATOM	748	C	ALA	C	62	-14.387	22.270	28.114	1.00	39.58	C
ATOM	749	O	ALA	C	62	-14.056	21.095	28.287	1.00	38.59	C
ATOM	750	N	PRO	C	63	-15.105	22.946	29.029	1.00	43.28	C
ATOM	751	CD	PRO	C	63	-15.305	24.404	29.056	1.00	43.94	C
ATOM	752	CA	PRO	C	63	-15.559	22.347	30.292	1.00	45.95	C
ATOM	753	CB	PRO	C	63	-16.002	23.558	31.116	1.00	46.29	C
ATOM	754	CG	PRO	C	63	-15.224	24.694	30.529	1.00	45.50	C
ATOM	755	C	PRO	C	63	-16.687	21.319	30.182	1.00	48.67	C
ATOM	756	O	PRO	C	63	-17.376	21.235	29.166	1.00	48.01	C
ATOM	757	N	LEU	C	64	-16.855	20.568	31.272	1.00	52.88	C
ATOM	758	CA	LEU	C	64	-17.870	19.528	31.463	1.00	55.50	C
ATOM	759	CB	LEU	C	64	-19.061	19.715	30.522	1.00	56.62	C
ATOM	760	CG	LEU	C	64	-19.808	21.040	30.660	1.00	58.22	C
ATOM	761	CD1	LEU	C	64	-21.293	20.790	30.430	1.00	59.37	C
ATOM	762	CD2	LEU	C	64	-19.580	21.635	32.040	1.00	58.04	C
ATOM	763	C	LEU	C	64	-17.324	18.127	31.304	1.00	57.11	C
ATOM	764	OT1	LEU	C	64	-16.093	17.996	31.140	1.00	58.74	C
ATOM	765	OXT	LEU	C	64	-18.143	17.178	31.358	1.00	57.70	C
ATOM	766	OH2	WAT	W	1	-13.420	23.772	13.420	1.00	68.77	W
ATOM	767	OH2	WAT	W	2	5.123	4.904	12.841	1.00	49.26	W
ATOM	768	OH2	WAT	W	3	-9.710	8.110	16.167	1.00	45.79	W
ATOM	769	OH2	WAT	W	4	3.964	25.988	25.041	1.00	54.34	W
ATOM	770	OH2	WAT	W	5	9.154	10.467	18.403	1.00	53.88	W
ATOM	771	OH2	WAT	W	6	-1.831	38.943	35.406	1.00	67.81	W
ATOM	772	OH2	WAT	W	7	-3.143	7.399	14.215	1.00	29.35	W
ATOM	773	OH2	WAT	W	8	-14.590	17.960	14.582	1.00	46.20	W
ATOM	774	OH2	WAT	W	9	-2.241	21.870	7.572	1.00	48.21	W
ATOM	775	OH2	WAT	W	10	5.133	17.056	23.108	1.00	30.14	W
ATOM	776	OH2	WAT	W	11	2.633	16.411	28.461	1.00	38.79	W
ATOM	777	OH2	WAT	W	12	-2.644	31.927	18.931	1.00	37.23	W
ATOM	778	OH2	WAT	W	13	3.849	23.009	26.307	1.00	55.69	W
ATOM	779	OH2	WAT	W	14	-7.124	5.979	19.437	1.00	36.83	W
ATOM	780	OH2	WAT	W	15	9.980	5.519	30.788	1.00	46.86	W
ATOM	781	OH2	WAT	W	16	-10.231	17.718	13.498	1.00	26.29	W
ATOM	782	OH2	WAT	W	17	-4.408	10.918	24.751	1.00	29.96	W
ATOM	783	OH2	WAT	W	18	7.236	20.067	23.369	1.00	42.55	W
ATOM	784	OH2	WAT	W	19	3.084	15.212	24.509	1.00	46.38	W
ATOM	785	OH2	WAT	W	20	-8.400	32.856	29.980	1.00	60.13	W
ATOM	786	OH2	WAT	W	21	2.278	1.311	19.768	1.00	44.76	W
ATOM	787	OH2	WAT	W	22	-5.002	30.025	18.420	1.00	36.23	W
ATOM	788	OH2	WAT	W	23	-1.894	1.792	15.573	1.00	42.85	W
ATOM	789	OH2	WAT	W	24	2.515	20.323	12.519	1.00	42.58	W
ATOM	790	OH2	WAT	W	25	-1.706	13.946	10.623	1.00	48.03	W
ATOM	791	OH2	WAT	W	26	-7.512	8.258	24.298	1.00	34.80	W
ATOM	792	OH2	WAT	W	27	-13.597	3.240	18.060	1.00	44.21	W
ATOM	793	OH2	WAT	W	28	8.250	19.107	15.955	1.00	48.54	W
ATOM	794	OH2	WAT	W	29	6.930	10.297	13.784	1.00	49.24	W
ATOM	795	OH2	WAT	W	30	8.076	20.627	32.202	1.00	66.78	W
ATOM	796	OH2	WAT	W	31	0.454	-3.723	20.999	1.00	44.55	W
ATOM	797	OH2	WAT	W	32	-8.565	6.277	22.112	1.00	34.63	W
ATOM	798	OH2	WAT	W	33	8.280	18.689	11.682	1.00	78.53	W
ATOM	799	OH2	WAT	W	34	-8.092	0.771	14.789	1.00	47.22	W
ATOM	800	OH2	WAT	W	35	-6.959	25.238	10.263	1.00	54.50	W
ATOM	801	OH2	WAT	W	36	6.625	0.437	15.541	1.00	61.08	W
ATOM	802	OH2	WAT	W	37	-12.355	24.838	12.355	1.00	34.01	W
ATOM	803	OH2	WAT	W	38	8.509	2.752	16.695	1.00	59.59	W
ATOM	804	OH2	WAT	W	39	4.162	17.600	11.512	1.00	48.15	W

Fig. 5 (contd.)

ATOM	805	OH2	WAT	W	40	1.920	-3.265	24.783	1.00	57.34	W
ATOM	806	OH2	WAT	W	41	-0.597	19.777	9.560	1.00	40.19	W
ATOM	807	OH2	WAT	W	42	-3.808	24.960	7.754	1.00	67.71	W
ATOM	808	OH2	WAT	W	43	-7.378	11.115	23.652	1.00	41.42	W
ATOM	809	OH2	WAT	W	44	-4.078	-0.471	16.534	1.00	76.11	W
ATOM	810	OH2	WAT	W	45	-6.606	27.261	12.681	1.00	48.25	W
ATOM	811	OH2	WAT	W	46	6.971	19.806	27.054	1.00	62.02	W
ATOM	812	OH2	WAT	W	47	-5.667	31.612	15.738	1.00	53.82	W
END											

Fig. 6

ATOM	234	CB	SER	B	39	-15.365	-2.991	19.079	1.00	59.00	B
ATOM	235	OG	SER	B	39	-14.721	-3.902	19.953	1.00	61.35	B
ATOM	236	C	SER	B	39	-13.310	-1.576	18.903	1.00	56.28	B
ATOM	237	O	SER	B	39	-12.244	-1.252	18.377	1.00	56.57	B
ATOM	238	N	SER	B	39	-13.720	-3.401	17.275	1.00	58.47	B
ATOM	239	CA	SER	B	39	-14.362	-2.351	18.115	1.00	57.58	B
ATOM	240	N	ALA	B	40	-13.611	-1.286	20.164	1.00	53.32	B
ATOM	241	CA	ALA	B	40	-12.690	-0.544	21.012	1.00	50.57	B
ATOM	242	CB	ALA	B	40	-13.348	-0.230	22.346	1.00	50.99	B
ATOM	243	C	ALA	B	40	-11.396	-1.314	21.239	1.00	49.80	B
ATOM	244	O	ALA	B	40	-11.406	-2.534	21.407	1.00	50.30	B
ATOM	245	N	LEU	B	41	-10.281	-0.591	21.237	1.00	45.57	B
ATOM	246	CA	LEU	B	41	-8.971	-1.193	21.454	1.00	43.28	B
ATOM	247	CB	LEU	B	41	-7.877	-0.227	20.990	1.00	40.27	B
ATOM	248	CG	LEU	B	41	-7.494	-0.187	19.501	1.00	40.88	B
ATOM	249	CD1	LEU	B	41	-8.630	-0.665	18.610	1.00	37.72	B
ATOM	250	CD2	LEU	B	41	-7.069	1.232	19.143	1.00	37.68	B
ATOM	251	C	LEU	B	41	-8.783	-1.539	22.932	1.00	42.24	B
ATOM	252	O	LEU	B	41	-9.236	-0.808	23.814	1.00	41.16	B
ATOM	253	N	ALA	B	42	-8.113	-2.655	23.200	1.00	41.11	B
ATOM	254	CA	ALA	B	42	-7.891	-3.093	24.576	1.00	40.48	B
ATOM	255	CB	ALA	B	42	-7.864	-4.618	24.639	1.00	39.75	B
ATOM	256	C	ALA	B	42	-6.610	-2.524	25.169	1.00	39.24	B
ATOM	257	O	ALA	B	42	-5.830	-1.868	24.477	1.00	38.76	B
ATOM	258	N	GLU	B	43	-6.403	-2.771	26.460	1.00	39.27	B
ATOM	259	CA	GLU	B	43	-5.213	-2.288	27.150	1.00	38.65	B
ATOM	260	CB	GLU	B	43	-5.316	-2.573	28.655	1.00	41.85	B
ATOM	261	CG	GLU	B	43	-4.159	-1.998	29.472	1.00	45.40	B
ATOM	262	CD	GLU	B	43	-4.231	-2.364	30.948	1.00	47.82	B
ATOM	263	OE1	GLU	B	43	-5.227	-2.012	31.614	1.00	50.74	B
ATOM	264	OE2	GLU	B	43	-3.283	-3.006	31.444	1.00	49.75	B
ATOM	265	C	GLU	B	43	-3.965	-2.962	26.586	1.00	37.56	B
ATOM	266	O	GLU	B	43	-3.904	-4.188	26.486	1.00	37.11	B
ATOM	267	N	GLY	B	44	-2.974	-2.156	26.218	1.00	35.48	B
ATOM	268	CA	GLY	B	44	-1.740	-2.691	25.670	1.00	33.93	B
ATOM	269	C	GLY	B	44	-1.711	-2.703	24.152	1.00	32.93	B
ATOM	270	O	GLY	B	44	-0.666	-2.932	23.542	1.00	32.61	B
ATOM	271	N	GLN	B	45	-2.859	-2.446	23.535	1.00	31.74	B
ATOM	272	CA	GLN	B	45	-2.949	-2.447	22.081	1.00	30.82	B
ATOM	273	CB	GLN	B	45	-4.373	-2.818	21.653	1.00	31.47	B
ATOM	274	CG	GLN	B	45	-4.530	-3.118	20.164	1.00	31.16	B
ATOM	275	CD	GLN	B	45	-5.929	-3.615	19.810	1.00	30.41	B
ATOM	276	OE1	GLN	B	45	-6.200	-3.967	18.664	1.00	30.09	B
ATOM	277	NE2	GLN	B	45	-6.816	-3.650	20.797	1.00	29.72	B
ATOM	278	C	GLN	B	45	-2.556	-1.097	21.481	1.00	30.79	B
ATOM	279	O	GLN	B	45	-2.806	-0.045	22.067	1.00	31.74	B
ATOM	280	N	SER	B	46	-1.930	-1.127	20.310	1.00	29.55	B
ATOM	281	CA	SER	B	46	-1.529	0.107	19.647	1.00	29.84	B
ATOM	282	CB	SER	B	46	-0.772	-0.208	18.358	1.00	28.36	B
ATOM	283	OG	SER	B	46	0.549	-0.617	18.664	1.00	34.06	B
ATOM	284	C	SER	B	46	-2.750	0.975	19.347	1.00	27.63	B
ATOM	285	O	SER	B	46	-3.851	0.467	19.134	1.00	26.91	B
ATOM	286	N	CYS	B	47	-2.543	2.287	19.332	1.00	27.18	B
ATOM	287	CA	CYS	B	47	-3.619	3.233	19.083	1.00	25.85	B
ATOM	288	C	CYS	B	47	-3.033	4.544	18.572	1.00	26.46	B
ATOM	289	O	CYS	B	47	-1.831	4.790	18.685	1.00	28.40	B

Fig. 6 (contd.)

ATOM	290	CB	CYS	B	47	-4.388	3.499	20.389	1.00	25.30	B
ATOM	291	SG	CYS	B	47	-3.300	4.101	21.724	1.00	28.90	B
ATOM	292	N	GLY	B	48	-3.895	5.389	18.018	1.00	25.42	B
ATOM	293	CA	GLY	B	48	-3.441	6.670	17.518	1.00	24.68	B
ATOM	294	C	GLY	B	48	-4.594	7.619	17.293	1.00	24.57	B
ATOM	295	O	GLY	B	48	-5.754	7.265	17.497	1.00	24.39	B
ATOM	296	N	VAL	B	49	-4.276	8.832	16.866	1.00	25.01	B
ATOM	297	CA	VAL	B	49	-5.297	9.833	16.599	1.00	24.08	B
ATOM	298	CB	VAL	B	49	-4.652	11.139	16.072	1.00	23.73	B
ATOM	299	CG1	VAL	B	49	-5.724	12.123	15.636	1.00	23.71	B
ATOM	300	CG2	VAL	B	49	-3.784	11.748	17.150	1.00	21.54	B
ATOM	301	C	VAL	B	49	-6.320	9.349	15.576	1.00	25.55	B
ATOM	302	O	VAL	B	49	-7.507	9.628	15.701	1.00	27.63	B
ATOM	303	N	TYR	B	50	-5.859	8.603	14.577	1.00	27.40	B
ATOM	304	CA	TYR	B	50	-6.735	8.131	13.510	1.00	28.08	B
ATOM	305	CB	TYR	B	50	-6.067	8.426	12.168	1.00	26.58	B
ATOM	306	CG	TYR	B	50	-5.626	9.868	12.032	1.00	26.76	B
ATOM	307	CD1	TYR	B	50	-6.561	10.888	11.835	1.00	24.56	B
ATOM	308	CE1	TYR	B	50	-6.164	12.218	11.736	1.00	23.17	B
ATOM	309	CD2	TYR	B	50	-4.274	10.218	12.127	1.00	23.57	B
ATOM	310	CE2	TYR	B	50	-3.866	11.546	12.030	1.00	24.76	B
ATOM	311	CZ	TYR	B	50	-4.817	12.541	11.834	1.00	19.86	B
ATOM	312	OH	TYR	B	50	-4.420	13.854	11.736	1.00	20.16	B
ATOM	313	C	TYR	B	50	-7.173	6.663	13.549	1.00	29.31	B
ATOM	314	O	TYR	B	50	-7.685	6.148	12.556	1.00	31.59	B
ATOM	315	N	THR	B	51	-6.981	5.991	14.679	1.00	29.31	B
ATOM	316	CA	THR	B	51	-7.384	4.591	14.800	1.00	28.76	B
ATOM	317	CB	THR	B	51	-6.333	3.742	15.566	1.00	27.30	B
ATOM	318	OG1	THR	B	51	-6.285	4.165	16.935	1.00	27.10	B
ATOM	319	CG2	THR	B	51	-4.944	3.896	14.944	1.00	25.06	B
ATOM	320	C	THR	B	51	-8.672	4.545	15.600	1.00	30.25	B
ATOM	321	O	THR	B	51	-9.198	5.584	15.997	1.00	29.66	B
ATOM	322	N	GLU	B	52	-9.187	3.343	15.832	1.00	32.77	B
ATOM	323	CA	GLU	B	52	-10.393	3.198	16.634	1.00	35.03	B
ATOM	324	CB	GLU	B	52	-10.827	1.730	16.707	1.00	38.95	B
ATOM	325	CG	GLU	B	52	-11.317	1.146	15.395	1.00	42.98	B
ATOM	326	CD	GLU	B	52	-12.554	1.852	14.872	1.00	48.93	B
ATOM	327	OE1	GLU	B	52	-13.516	2.021	15.653	1.00	49.81	B
ATOM	328	OE2	GLU	B	52	-12.566	2.233	13.680	1.00	49.71	B
ATOM	329	C	GLU	B	52	-9.995	3.682	18.021	1.00	34.86	B
ATOM	330	O	GLU	B	52	-8.806	3.718	18.356	1.00	32.99	B
ATOM	331	N	ARG	B	53	-10.977	4.050	18.831	1.00	36.33	B
ATOM	332	CA	ARG	B	53	-10.691	4.528	20.174	1.00	38.61	B
ATOM	333	CB	ARG	B	53	-11.853	5.389	20.675	1.00	43.21	B
ATOM	334	CG	ARG	B	53	-11.988	6.685	19.897	1.00	49.72	B
ATOM	335	CD	ARG	B	53	-13.194	7.500	20.324	1.00	56.57	B
ATOM	336	NE	ARG	B	53	-13.145	7.890	21.729	1.00	61.53	B
ATOM	337	CZ	ARG	B	53	-13.987	8.753	22.288	1.00	63.65	B
ATOM	338	NH1	ARG	B	53	-14.939	9.316	21.556	1.00	64.66	B
ATOM	339	NH2	ARG	B	53	-13.878	9.054	23.575	1.00	65.21	B
ATOM	340	C	ARG	B	53	-10.412	3.397	21.153	1.00	37.36	B
ATOM	341	O	ARG	B	53	-10.854	2.263	20.953	1.00	37.28	B
ATOM	342	N	CYS	B	54	-9.655	3.711	22.201	1.00	35.80	B
ATOM	343	CA	CYS	B	54	-9.324	2.733	23.232	1.00	37.19	B
ATOM	344	C	CYS	B	54	-10.600	2.430	24.001	1.00	39.75	B
ATOM	345	O	CYS	B	54	-11.555	3.205	23.949	1.00	39.46	B
ATOM	346	CB	CYS	B	54	-8.283	3.289	24.210	1.00	32.76	B
ATOM	347	SG	CYS	B	54	-6.595	3.539	23.563	1.00	31.75	B

Fig. 6 (contd.)

ATOM	348	N	ALA	B	55	-10.612	1.311	24.722	1.00	39.90	B
ATOM	349	CA	ALA	B	55	-11.782	0.929	25.496	1.00	42.01	B
ATOM	350	CB	ALA	B	55	-11.620	-0.493	26.035	1.00	40.89	B
ATOM	351	C	ALA	B	55	-11.980	1.909	26.643	1.00	43.33	B
ATOM	352	O	ALA	B	55	-11.035	2.562	27.090	1.00	44.41	B
ATOM	353	N	GLN	B	56	-13.217	2.005	27.114	1.00	46.27	B
ATOM	354	CA	GLN	B	56	-13.569	2.900	28.209	1.00	46.98	B
ATOM	355	CB	GLN	B	56	-15.020	2.661	28.631	1.00	49.45	B
ATOM	356	CG	GLN	B	56	-16.030	2.703	27.487	1.00	52.92	B
ATOM	357	CD	GLN	B	56	-16.073	1.414	26.671	1.00	55.64	B
ATOM	358	OE1	GLN	B	56	-15.076	0.999	26.077	1.00	54.71	B
ATOM	359	NE2	GLN	B	56	-17.240	0.777	26.641	1.00	55.29	B
ATOM	360	C	GLN	B	56	-12.649	2.685	29.405	1.00	46.86	B
ATOM	361	O	GLN	B	56	-12.315	1.549	29.746	1.00	46.71	B
ATOM	362	N	GLY	B	57	-12.242	3.779	30.040	1.00	46.46	B
ATOM	363	CA	GLY	B	57	-11.366	3.679	31.193	1.00	45.89	B
ATOM	364	C	GLY	B	57	-9.903	3.715	30.799	1.00	46.63	B
ATOM	365	O	GLY	B	57	-9.017	3.747	31.653	1.00	47.54	B
ATOM	366	N	LEU	B	58	-9.650	3.707	29.496	1.00	45.76	B
ATOM	367	CA	LEU	B	58	-8.290	3.745	28.974	1.00	44.32	B
ATOM	368	CB	LEU	B	58	-7.982	2.450	28.212	1.00	45.02	B
ATOM	369	CG	LEU	B	58	-8.134	1.093	28.908	1.00	45.27	B
ATOM	370	CD1	LEU	B	58	-8.043	-0.017	27.872	1.00	44.01	B
ATOM	371	CD2	LEU	B	58	-7.060	0.928	29.970	1.00	44.12	B
ATOM	372	C	LEU	B	58	-8.153	4.923	28.009	1.00	43.40	B
ATOM	373	O	LEU	B	58	-9.143	5.390	27.441	1.00	42.14	B
ATOM	374	N	ARG	B	59	-6.929	5.407	27.836	1.00	40.99	B
ATOM	375	CA	ARG	B	59	-6.674	6.487	26.899	1.00	41.20	B
ATOM	376	CB	ARG	B	59	-6.593	7.853	27.604	1.00	42.80	B
ATOM	377	CG	ARG	B	59	-5.456	8.032	28.582	1.00	48.53	B
ATOM	378	CD	ARG	B	59	-5.366	9.484	29.058	1.00	50.94	B
ATOM	379	NE	ARG	B	59	-5.012	10.404	27.977	1.00	54.00	B
ATOM	380	CZ	ARG	B	59	-4.944	11.728	28.108	1.00	55.67	B
ATOM	381	NH1	ARG	B	59	-5.208	12.300	29.276	1.00	55.62	B
ATOM	382	NH2	ARG	B	59	-4.607	12.485	27.070	1.00	54.65	B
ATOM	383	C	ARG	B	59	-5.382	6.183	26.149	1.00	38.98	B
ATOM	384	O	ARG	B	59	-4.509	5.465	26.648	1.00	37.23	B
ATOM	385	N	CYS	B	60	-5.277	6.724	24.941	1.00	34.30	B
ATOM	386	CA	CYS	B	60	-4.121	6.504	24.097	1.00	34.00	B
ATOM	387	C	CYS	B	60	-2.931	7.400	24.451	1.00	34.57	B
ATOM	388	O	CYS	B	60	-2.995	8.617	24.305	1.00	34.82	B
ATOM	389	CB	CYS	B	60	-4.527	6.698	22.630	1.00	31.12	B
ATOM	390	SG	CYS	B	60	-3.259	6.122	21.472	1.00	28.31	B
ATOM	391	N	LEU	B	61	-1.846	6.785	24.919	1.00	35.17	B
ATOM	392	CA	LEU	B	61	-0.637	7.516	25.295	1.00	37.27	B
ATOM	393	CB	LEU	B	61	-0.345	7.379	26.794	1.00	36.88	B
ATOM	394	CG	LEU	B	61	-1.281	8.031	27.804	1.00	39.20	B
ATOM	395	CD1	LEU	B	61	-1.388	9.534	27.548	1.00	37.47	B
ATOM	396	CD2	LEU	B	61	-2.625	7.367	27.702	1.00	40.69	B
ATOM	397	C	LEU	B	61	0.571	7.005	24.539	1.00	37.81	B
ATOM	398	O	LEU	B	61	0.640	5.830	24.187	1.00	37.72	B
ATOM	399	N	PRO	B	62	1.553	7.885	24.293	1.00	40.67	B
ATOM	400	CD	PRO	B	62	1.530	9.335	24.565	1.00	42.50	B
ATOM	401	CA	PRO	B	62	2.771	7.512	23.573	1.00	43.32	B
ATOM	402	CB	PRO	B	62	3.245	8.843	23.011	1.00	42.71	B
ATOM	403	CG	PRO	B	62	2.918	9.781	24.133	1.00	41.70	B
ATOM	404	C	PRO	B	62	3.806	6.884	24.502	1.00	45.58	B
ATOM	405	O	PRO	B	62	3.670	6.939	25.723	1.00	43.82	B

Fig. 6 (contd.)

ATOM	406	N	ARG	B	63	4.834	6.283	23.913	1.00	48.66	B
ATOM	407	CA	ARG	B	63	5.910	5.663	24.678	1.00	52.24	B
ATOM	408	CB	ARG	B	63	6.805	4.830	23.757	1.00	54.66	B
ATOM	409	CG	ARG	B	63	6.073	4.003	22.705	1.00	57.98	B
ATOM	410	CD	ARG	B	63	5.569	2.675	23.239	1.00	60.28	B
ATOM	411	NE	ARG	B	63	5.043	1.841	22.159	1.00	63.63	B
ATOM	412	CZ	ARG	B	63	4.710	0.561	22.294	1.00	64.74	B
ATOM	413	NH1	ARG	B	63	4.847	-0.043	23.467	1.00	64.62	B
ATOM	414	NH2	ARG	B	63	4.239	-0.116	21.254	1.00	64.94	B
ATOM	415	C	ARG	B	63	6.731	6.811	25.261	1.00	53.02	B
ATOM	416	O	ARG	B	63	7.001	7.790	24.565	1.00	52.38	B
ATOM	417	N	GLN	B	64	7.132	6.694	26.524	1.00	54.59	B
ATOM	418	CA	GLN	B	64	7.918	7.746	27.161	1.00	56.19	B
ATOM	419	CB	GLN	B	64	8.112	7.454	28.654	1.00	58.46	B
ATOM	420	CG	GLN	B	64	6.844	7.599	29.483	1.00	62.16	B
ATOM	421	CD	GLN	B	64	7.117	7.649	30.977	1.00	64.31	B
ATOM	422	OE1	GLN	B	64	6.189	7.677	31.787	1.00	65.56	B
ATOM	423	NE2	GLN	B	64	8.393	7.669	31.349	1.00	65.48	B
ATOM	424	C	GLN	B	64	9.278	7.975	26.508	1.00	54.95	B
ATOM	425	O	GLN	B	64	9.976	8.924	26.852	1.00	55.19	B
ATOM	426	N	ASP	B	65	9.653	7.117	25.566	1.00	54.23	B
ATOM	427	CA	ASP	B	65	10.936	7.273	24.888	1.00	54.67	B
ATOM	428	CB	ASP	B	65	11.622	5.914	24.714	1.00	56.29	B
ATOM	429	CG	ASP	B	65	10.868	4.995	23.776	1.00	57.63	B
ATOM	430	OD1	ASP	B	65	9.738	4.586	24.119	1.00	57.64	B
ATOM	431	OD2	ASP	B	65	11.407	4.684	22.693	1.00	58.07	B
ATOM	432	C	ASP	B	65	10.795	7.952	23.524	1.00	54.03	B
ATOM	433	O	ASP	B	65	11.772	8.089	22.786	1.00	54.88	B
ATOM	434	N	GLU	B	66	9.581	8.376	23.188	1.00	52.05	B
ATOM	435	CA	GLU	B	66	9.346	9.044	21.912	1.00	50.06	B
ATOM	436	CB	GLU	B	66	7.841	9.145	21.629	1.00	47.94	B
ATOM	437	CG	GLU	B	66	7.164	7.825	21.284	1.00	45.31	B
ATOM	438	CD	GLU	B	66	7.504	7.334	19.885	1.00	44.11	B
ATOM	439	OE1	GLU	B	66	8.316	7.987	19.197	1.00	44.31	B
ATOM	440	OE2	GLU	B	66	6.958	6.291	19.468	1.00	43.74	B
ATOM	441	C	GLU	B	66	9.952	10.443	21.938	1.00	49.71	B
ATOM	442	O	GLU	B	66	9.778	11.183	22.905	1.00	49.58	B
ATOM	443	N	GLU	B	67	10.668	10.799	20.876	1.00	49.99	B
ATOM	444	CA	GLU	B	67	11.275	12.123	20.775	1.00	50.53	B
ATOM	445	CB	GLU	B	67	12.245	12.170	19.591	1.00	53.83	B
ATOM	446	CG	GLU	B	67	13.195	10.979	19.489	1.00	59.06	B
ATOM	447	CD	GLU	B	67	14.234	10.942	20.595	1.00	62.91	B
ATOM	448	OE1	GLU	B	67	13.845	10.864	21.780	1.00	64.79	B
ATOM	449	OE2	GLU	B	67	15.443	10.987	20.276	1.00	64.81	B
ATOM	450	C	GLU	B	67	10.139	13.125	20.547	1.00	48.30	B
ATOM	451	O	GLU	B	67	10.140	14.229	21.092	1.00	47.77	B
ATOM	452	N	LYS	B	68	9.170	12.715	19.733	1.00	44.89	B
ATOM	453	CA	LYS	B	68	8.010	13.537	19.404	1.00	41.91	B
ATOM	454	CB	LYS	B	68	7.980	13.821	17.903	1.00	44.05	B
ATOM	455	CG	LYS	B	68	9.325	14.192	17.299	1.00	48.60	B
ATOM	456	CD	LYS	B	68	9.857	15.501	17.840	1.00	51.03	B
ATOM	457	CE	LYS	B	68	11.163	15.858	17.156	1.00	54.19	B
ATOM	458	NZ	LYS	B	68	11.008	15.877	15.673	1.00	54.98	B
ATOM	459	C	LYS	B	68	6.757	12.753	19.786	1.00	36.81	B
ATOM	460	O	LYS	B	68	6.151	12.096	18.944	1.00	36.07	B
ATOM	461	N	PRO	B	69	6.352	12.814	21.064	1.00	33.10	B
ATOM	462	CD	PRO	B	69	6.900	13.675	22.126	1.00	31.55	B
ATOM	463	CA	PRO	B	69	5.166	12.094	21.540	1.00	30.73	B

Fig. 6 (contd.)

ATOM	464	CB	PRO	B	69	5.032	12.557	22.993	1.00	29.25	B
ATOM	465	CG	PRO	B	69	6.414	12.979	23.366	1.00	33.62	B
ATOM	466	C	PRO	B	69	3.895	12.383	20.738	1.00	28.46	B
ATOM	467	O	PRO	B	69	3.145	11.466	20.407	1.00	26.39	B
ATOM	468	N	LEU	B	70	3.647	13.657	20.445	1.00	26.72	B
ATOM	469	CA	LEU	B	70	2.447	14.033	19.700	1.00	27.00	B
ATOM	470	CB	LEU	B	70	2.311	15.559	19.632	1.00	25.43	B
ATOM	471	CG	LEU	B	70	2.028	16.231	20.981	1.00	25.63	B
ATOM	472	CD1	LEU	B	70	1.938	17.740	20.801	1.00	26.23	B
ATOM	473	CD2	LEU	B	70	0.727	15.684	21.565	1.00	23.13	B
ATOM	474	C	LEU	B	70	2.485	13.438	18.306	1.00	26.72	B
ATOM	475	O	LEU	B	70	1.488	12.881	17.829	1.00	25.47	B
ATOM	476	N	HIS	B	71	3.638	13.545	17.653	1.00	26.27	B
ATOM	477	CA	HIS	B	71	3.787	12.978	16.322	1.00	26.43	B
ATOM	478	CB	HIS	B	71	5.129	13.384	15.702	1.00	26.14	B
ATOM	479	CG	HIS	B	71	5.135	14.777	15.153	1.00	28.44	B
ATOM	480	CD2	HIS	B	71	4.945	15.236	13.893	1.00	29.08	B
ATOM	481	ND1	HIS	B	71	5.291	15.893	15.947	1.00	28.51	B
ATOM	482	CE1	HIS	B	71	5.196	16.979	15.201	1.00	28.30	B
ATOM	483	NE2	HIS	B	71	4.985	16.607	13.951	1.00	30.59	B
ATOM	484	C	HIS	B	71	3.654	11.457	16.381	1.00	25.51	B
ATOM	485	O	HIS	B	71	3.177	10.841	15.434	1.00	26.07	B
ATOM	486	N	ALA	B	72	4.048	10.853	17.499	1.00	25.45	B
ATOM	487	CA	ALA	B	72	3.939	9.400	17.643	1.00	26.65	B
ATOM	488	CB	ALA	B	72	4.620	8.936	18.927	1.00	26.26	B
ATOM	489	C	ALA	B	72	2.467	8.996	17.660	1.00	27.13	B
ATOM	490	O	ALA	B	72	2.083	7.976	17.086	1.00	26.66	B
ATOM	491	N	LEU	B	73	1.650	9.805	18.327	1.00	26.56	B
ATOM	492	CA	LEU	B	73	0.219	9.552	18.423	1.00	27.39	B
ATOM	493	CB	LEU	B	73	-0.412	10.502	19.442	1.00	30.38	B
ATOM	494	CG	LEU	B	73	-0.845	9.899	20.780	1.00	35.02	B
ATOM	495	CD1	LEU	B	73	0.235	8.981	21.334	1.00	37.66	B
ATOM	496	CD2	LEU	B	73	-1.146	11.031	21.749	1.00	34.89	B
ATOM	497	C	LEU	B	73	-0.458	9.717	17.069	1.00	24.51	B
ATOM	498	O	LEU	B	73	-1.336	8.939	16.703	1.00	24.68	B
ATOM	499	N	LEU	B	74	-0.044	10.737	16.327	1.00	25.25	B
ATOM	500	CA	LEU	B	74	-0.598	10.988	15.005	1.00	23.89	B
ATOM	501	CB	LEU	B	74	-0.006	12.280	14.423	1.00	22.63	B
ATOM	502	CG	LEU	B	74	-0.503	13.563	15.113	1.00	24.73	B
ATOM	503	CD1	LEU	B	74	0.428	14.725	14.804	1.00	24.97	B
ATOM	504	CD2	LEU	B	74	-1.937	13.864	14.663	1.00	23.07	B
ATOM	505	C	LEU	B	74	-0.287	9.807	14.090	1.00	26.28	B
ATOM	506	O	LEU	B	74	-1.095	9.440	13.235	1.00	23.75	B
ATOM	507	N	HIS	B	75	0.879	9.198	14.290	1.00	26.07	B
ATOM	508	CA	HIS	B	75	1.289	8.071	13.468	1.00	30.78	B
ATOM	509	CB	HIS	B	75	2.789	8.173	13.178	1.00	33.64	B
ATOM	510	CG	HIS	B	75	3.162	9.419	12.438	1.00	38.80	B
ATOM	511	CD2	HIS	B	75	3.870	10.507	12.821	1.00	40.91	B
ATOM	512	ND1	HIS	B	75	2.718	9.683	11.160	1.00	41.10	B
ATOM	513	CE1	HIS	B	75	3.133	10.881	10.789	1.00	41.21	B
ATOM	514	NE2	HIS	B	75	3.834	11.403	11.779	1.00	44.19	B
ATOM	515	C	HIS	B	75	0.935	6.703	14.052	1.00	32.10	B
ATOM	516	O	HIS	B	75	1.539	5.695	13.698	1.00	33.73	B
ATOM	517	N	GLY	B	76	-0.051	6.681	14.943	1.00	32.63	B
ATOM	518	CA	GLY	B	76	-0.508	5.436	15.541	1.00	32.02	B
ATOM	519	C	GLY	B	76	0.471	4.625	16.374	1.00	34.41	B
ATOM	520	O	GLY	B	76	0.343	3.399	16.446	1.00	34.43	B
ATOM	521	N	ARG	B	77	1.434	5.285	17.013	1.00	32.88	B

Fig. 6 (contd.)

ATOM	522	CA	ARG	B	77	2.410	4.580	17.837	1.00	34.75	B
ATOM	523	CB	ARG	B	77	3.828	5.075	17.538	1.00	35.67	B
ATOM	524	CG	ARG	B	77	4.297	4.757	16.126	1.00	37.36	B
ATOM	525	CD	ARG	B	77	5.807	4.695	16.057	1.00	37.48	B
ATOM	526	NE	ARG	B	77	6.435	5.956	16.436	1.00	38.77	B
ATOM	527	CZ	ARG	B	77	6.610	6.985	15.613	1.00	38.84	B
ATOM	528	NH1	ARG	B	77	6.204	6.909	14.355	1.00	38.80	B
ATOM	529	NH2	ARG	B	77	7.202	8.090	16.047	1.00	38.47	B
ATOM	530	C	ARG	B	77	2.125	4.701	19.329	1.00	34.86	B
ATOM	531	O	ARG	B	77	2.996	4.458	20.161	1.00	36.41	B
ATOM	532	N	GLY	B	78	0.900	5.082	19.669	1.00	33.38	B
ATOM	533	CA	GLY	B	78	0.545	5.190	21.068	1.00	31.84	B
ATOM	534	C	GLY	B	78	0.087	3.823	21.546	1.00	31.67	B
ATOM	535	O	GLY	B	78	0.032	2.871	20.767	1.00	31.30	B
ATOM	536	N	VAL	B	79	-0.239	3.721	22.824	1.00	31.21	B
ATOM	537	CA	VAL	B	79	-0.711	2.466	23.393	1.00	33.83	B
ATOM	538	CB	VAL	B	79	0.443	1.700	24.101	1.00	36.30	B
ATOM	539	CG1	VAL	B	79	1.096	2.589	25.140	1.00	39.47	B
ATOM	540	CG2	VAL	B	79	-0.094	0.436	24.760	1.00	38.17	B
ATOM	541	C	VAL	B	79	-1.811	2.774	24.403	1.00	32.69	B
ATOM	542	O	VAL	B	79	-1.735	3.768	25.125	1.00	34.48	B
ATOM	543	N	CYS	B	80	-2.842	1.939	24.432	1.00	32.38	B
ATOM	544	CA	CYS	B	80	-3.942	2.129	25.365	1.00	34.85	B
ATOM	545	C	CYS	B	80	-3.486	1.739	26.768	1.00	37.39	B
ATOM	546	O	CYS	B	80	-3.119	0.591	27.005	1.00	35.79	B
ATOM	547	CB	CYS	B	80	-5.144	1.270	24.964	1.00	32.27	B
ATOM	548	SG	CYS	B	80	-5.879	1.648	23.342	1.00	34.08	B
ATOM	549	N	LEU	B	81	-3.509	2.700	27.689	1.00	42.01	B
ATOM	550	CA	LEU	B	81	-3.091	2.463	29.070	1.00	46.11	B
ATOM	551	CB	LEU	B	81	-1.729	3.114	29.337	1.00	46.36	B
ATOM	552	CG	LEU	B	81	-0.510	2.625	28.548	1.00	47.24	B
ATOM	553	CD1	LEU	B	81	0.680	3.510	28.871	1.00	47.79	B
ATOM	554	CD2	LEU	B	81	-0.206	1.174	28.885	1.00	45.42	B
ATOM	555	C	LEU	B	81	-4.095	2.988	30.093	1.00	49.50	B
ATOM	556	O	LEU	B	81	-5.128	3.556	29.741	1.00	48.23	B
ATOM	557	N	ASN	B	82	-3.750	2.790	31.363	1.00	54.82	B
ATOM	558	CA	ASN	B	82	-4.541	3.195	32.527	1.00	59.40	B
ATOM	559	CB	ASN	B	82	-3.602	3.443	33.711	1.00	61.17	B
ATOM	560	CG	ASN	B	82	-2.493	4.426	33.378	1.00	62.57	B
ATOM	561	OD1	ASN	B	82	-2.714	5.414	32.677	1.00	64.48	B
ATOM	562	ND2	ASN	B	82	-1.295	4.165	33.887	1.00	62.95	B
ATOM	563	C	ASN	B	82	-5.487	4.394	32.410	1.00	61.49	B
ATOM	564	O	ASN	B	82	-6.503	4.339	31.719	1.00	61.26	B
ATOM	565	N	GLU	B	83	-5.141	5.458	33.134	1.00	64.37	B
ATOM	566	CA	GLU	B	83	-5.910	6.700	33.202	1.00	66.99	B
ATOM	567	CB	GLU	B	83	-4.964	7.905	33.200	1.00	68.36	B
ATOM	568	CG	GLU	B	83	-3.745	7.748	34.100	1.00	70.54	B
ATOM	569	CD	GLU	B	83	-4.079	7.137	35.448	1.00	71.94	B
ATOM	570	OE1	GLU	B	83	-4.920	7.708	36.172	1.00	73.55	B
ATOM	571	OE2	GLU	B	83	-3.496	6.084	35.783	1.00	72.89	B
ATOM	572	C	GLU	B	83	-6.939	6.864	32.096	1.00	67.27	B
ATOM	573	OT1	GLU	B	83	-8.128	7.052	32.426	1.00	68.26	B
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END											

Fig. 7

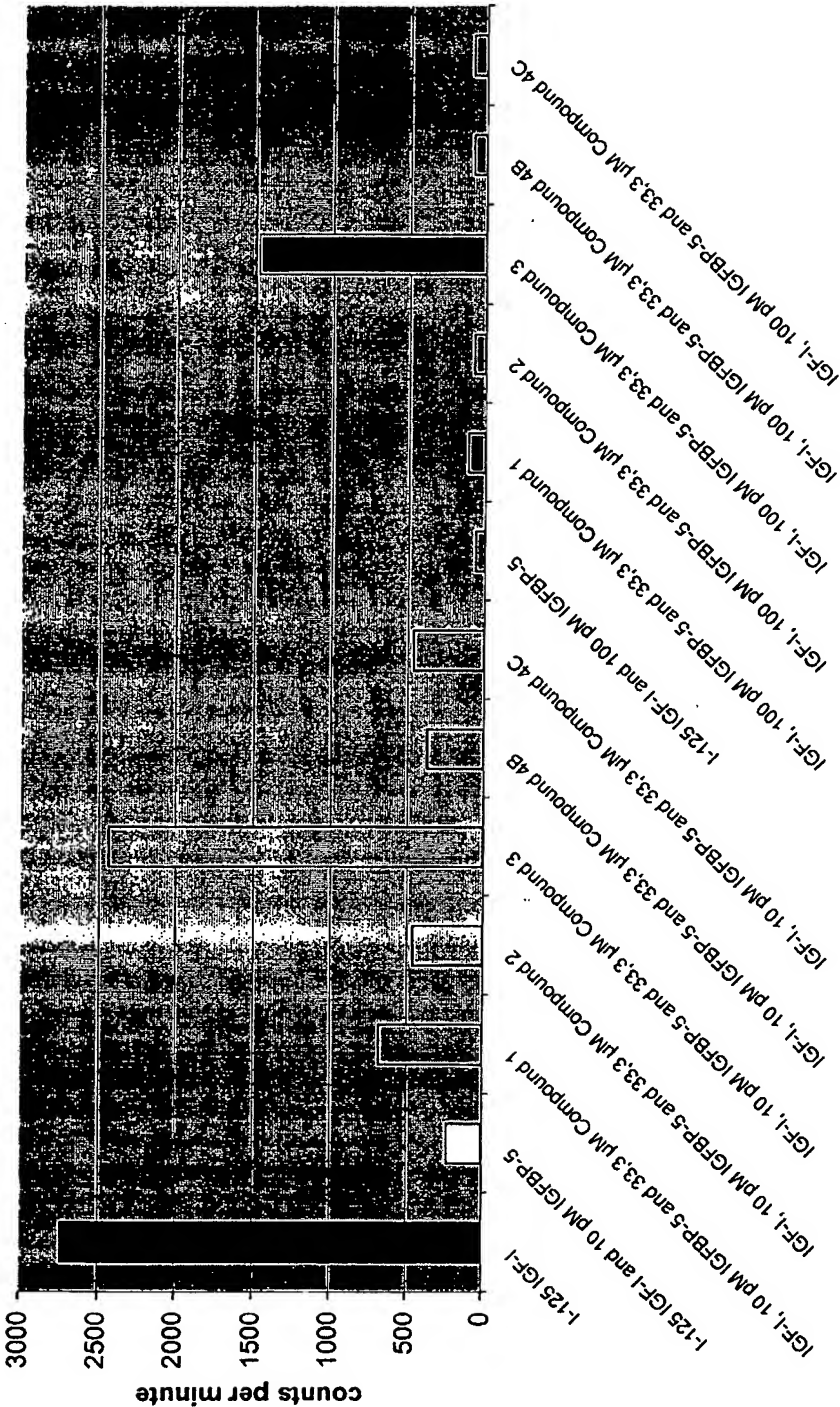
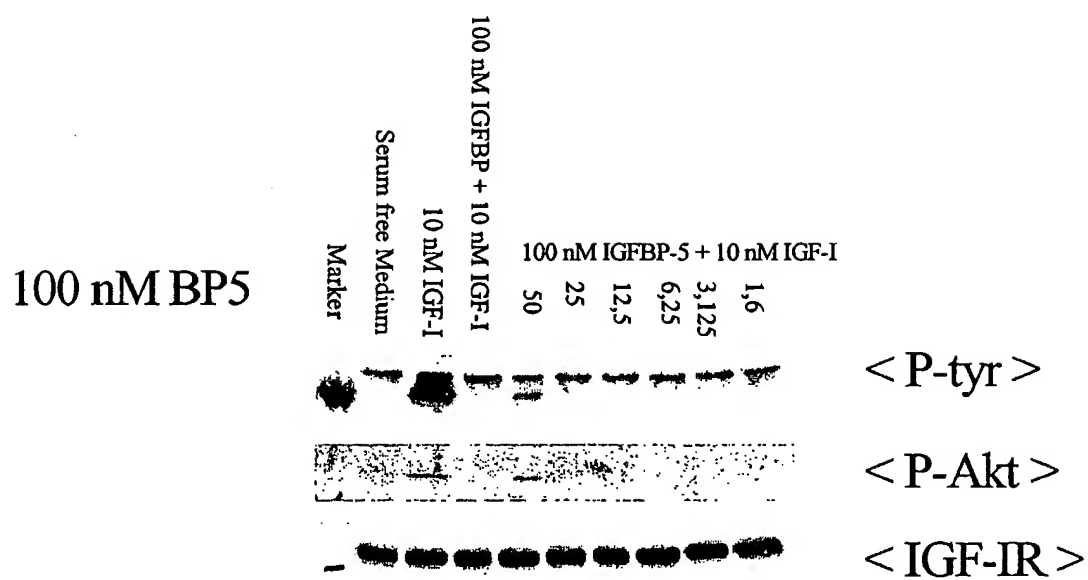


Fig. 8



IGFBP-5 and Compound 3

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